THE APPLICATION OF THE "METHOD OF MAXIMUM LIKELIHOOD" TO THE ESTIMATION OF LINKAGE

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INTRODUCTION

The "Method of Maximum Likelihood" is given by FISHER (1928) and is illustrated by a simple case of linkage between two factors. The method consists of multiplying each class frequency by the natural logarithm of its corresponding probability, summing, and determining the value for which the sum is a maximum. The linkage is expressed in terms of p where p is the proportion of gametes carrying both dominants plus the proportion of gametes carrying both recessives. The method is quite general, and may be applied to any type of ratio from which it is desired to estimate a linkage.

A CASE INVOLVING COMPLEMENTARY FACTORS

BRUNSON (1) has studied maize families in which a factor for pale green seedling is linked with one of two complementary factors for aleurone colour, and he gives a formula, derived from EMERSON'S formula, for the estimation of the linkage.

The F_2 is classified as under, where C and R are complementary factors for aleurone colour, and P_{g1} is a factor for pale green seedling:

TABLE 1

Frequencies observed in an F_2 segregating for aleurone colour and pale green seedling (BRUNSON'S *data*).

	CR	*Cr+cR+cr*	SEEDLING TOTAL
P_{g1}	1907	1053	2960
p_{g1}	300	686	986
Aleurone total	2207	1739	n=3946

Then, considering only the factor P_{g_1} and the aleurone factor linked with it, the probabilities of the four classes in F_2 will be:

$$\begin{array}{cccc} P_{g_1}A & p_{g_1}A & P_{g_1}a & p_{g_1}a \\ \frac{1}{4}(2+pp^1) : & \frac{1}{4}(1-pp^1) : & \frac{1}{4}(1-pp^1) : & \frac{1}{4}pp^1 \end{array}$$

where A is either C or R, and p is the proportion of $(P_{g_1}A + p_{g_1}a)$ gametes in male gametogenesis, and p^1 is the proportion of $(P_{g_1}A + p_{g_1}a)$ gametes in female gametogenesis. The effect of the linkage is then entirely expressed in the term pp^1, and if crossing over is equal in male and female, the cross-over percentage will be $(1-\sqrt{pp^1})\times 100$ in the coupling phase, and $(\sqrt{pp^1})\times 100$ in the repulsion phase. For simplicity, θ will be written for pp^1, and since the data provide no evidence on the matter, it will be assumed that crossing over is equal in male and female. (See FISHER (1928)).

Then, bringing in the complementary factor for aleurone colour, we get the probabilities in the four classes:

$$\begin{array}{cccc} CRP_{g_1} & CRp_{g_1} & [Cr+cR+cr]P_{g_1} & [Cr+cR+cr]p_{g_1} \\ \frac{3}{16}(2+\theta) & \frac{3}{16}(1-\theta) & \frac{3}{16}(2-\theta) & \frac{1}{16}(1+3\theta) \end{array}$$

Then the logarithm of the likelihood will be:

$$L = 1907 \log \frac{3}{16}(2+\theta) + 300 \log \frac{3}{16}(1-\theta) + 1053 \log \frac{3}{16}(2-\theta) + 686 \log \frac{1}{16}(1+3\theta). \qquad (1)$$

And the maximum likelihood value of θ will be that for which the first differential of equation 1, with respect to θ, is zero:

$$= \frac{1907}{2+\theta} - \frac{300}{1-\theta} - \frac{1053}{2-\theta} + \frac{2058}{1+3\theta} = 0. \qquad (2)$$

Which becomes on multiplying out:

$$11838\theta^3 - 12802\theta^2 - 11376\theta + 8740 = 0.$$

An equation of the third degree, which may be solved by HORNER'S method (BURNSIDE and PANTON 1886), or by the method of successive trials developed on page 532.

In the present case the solution is

$$\theta = 0.5902.$$

Then, assuming equal crossing over in male and female

$$p=\sqrt{\theta}=0.7682$$

or 23.18 percent crossing over, with coupling.

The variance of this estimate of θ may be obtained by differentiating equation 2 again with respect to θ, substituting the expected values for the class frequencies, and equating to $-1/V(\theta)$

$$-\frac{3n}{16}\cdot\frac{2+\theta}{(2+\theta)^2}-\frac{3n}{16}\cdot\frac{1-\theta}{(1-\theta)^2}-\frac{3n}{16}\cdot\frac{2-\theta}{(2-\theta)^2}-\frac{9n}{16}\cdot\frac{1+3\theta}{(1+3\theta)^2}=-\frac{1}{V(\theta)}$$

or

$$\frac{1}{V(\theta)}=\frac{3n}{16}\left(\frac{1}{2+\theta}+\frac{1}{1+\theta}+\frac{1}{2-\theta}+\frac{3}{1+3\theta}\right) \tag{3}$$

$$V(\theta)=\frac{4}{3n}\cdot\frac{(2+\theta)(1-\theta)(2-\theta)(1+3\theta)}{5+2\theta-4\theta^2}\,. \tag{4}$$

Then, since the variance of θ is the mean square deviation of all θ's from the mean of θ, and the variance of p is the mean square deviation of the p's from the mean of p, and since θ equals p^2, we can calculate the variance of p from the variance of θ.

$$V(\theta)=(2p)^2\cdot V(p)$$

and

$$V(p)=\frac{(2+p^2)(1-p^2)(2-p^2)(1+3p^2)}{3np^2(5+2p^2-4p^4)}\,. \tag{5}$$

Substituting for p we get

$$V(p)=0.0001240$$

$$\sigma(p)=0.011$$

BRUNSON'S formula may be reduced to

$$p_B{}^2=\frac{16}{18n}\cdot(a-b-c+3d)$$

where a, b, c, and d are the observed frequencies in the four classes, CRP_{g1}, CRp_{g1}, $[Cr+cR+cr]P_{g1}$, and $[Cr+cR+cr]p_{g1}$ respectively.

BRUNSON finds

$$p_B=0.767.$$

The variance of this estimate may be found from the general equation given by FISHER (1928).

$$\frac{1}{n}V(T)=S\left\{p\left(\frac{dT}{da}\right)^2\right\}-\left(\frac{dT}{dn}\right)^2 \tag{6}$$

where T is any function of the frequencies, and p is the probability of the corresponding class a, b, c, d. For convenience T_B may be used in place of $p_B{}^2$, and the variance of p_B found from that of T_B as above.

Then

$$T_B=\frac{16}{18n}(a-b-c+3d).$$

Taking the components of

$$S\left\{p\left(\frac{dT}{da}\right)^2\right\}$$

$$\frac{dT}{da}=\frac{16}{18n} \quad ; \qquad \left(\frac{dT}{da}\right)^2=\frac{64}{81n^2}$$

$$\frac{dT}{db}=-\frac{16}{18n}; \qquad \left(\frac{dT}{db}\right)^2=\frac{64}{81n^2}$$

$$\frac{dT}{dc}=-\frac{16}{18n}; \qquad \left(\frac{dT}{dc}\right)^2=\frac{64}{81n^2}$$

$$\frac{dT}{dd}=\frac{48}{18n} \quad ; \qquad \left(\frac{dT}{dd}\right)^2=\frac{64}{9n^2}$$

and

$$S\left\{p\left(\frac{dT}{da}\right)^2\right\}=(pa+pb+pc+9pd)\frac{64}{81n^2}$$

$$=\frac{24(1+\theta)}{16}\times\frac{64}{81n^2}=\frac{32(1+\theta)}{27n^2}.$$

And the component

$$\left(\frac{dT}{dn}\right)^2=\left(-\frac{16(a-b-c+3d)}{18n^2}\right)^2$$

$$=\left(\frac{-18n\theta}{18n^2}\right)^2=\frac{\theta^2}{n^2}$$

Then

$$\frac{1}{n}V(T_B)=\frac{32+32\theta-27\theta^2}{27n^2}$$

$$V(T_B)=\frac{32+32\theta-27\theta^2}{27n}$$

Then since

$$T_B = p_B^2 \; ; \quad V(p_B) = \frac{V(T_B)}{4/22}$$

as before, and

$$V(p_B) = \frac{32+32p^2-27p^4}{108np^2} .$$

Substituting the most likely value of p we get

$$V(p_B) = 0.000165$$
$$\sigma(p_B) = 0.013.$$

The efficiency of BRUNSON'S formula may be estimated by dividing the variance of the Maximum Likelihood equation by the variance of BRUNSON'S formula.

$$E = \frac{(2+p^2)(1-p^2)(2-p^2)(1+3p^2)}{3np^2(5+2p^2-4p^4)} \div \frac{32+32p^2-27p^4}{108np^2}$$
$$= \frac{36(2+p^2)(1-p^2)(2-p^2)(1+3p^2)}{(5+2p^2-4p^4)(32+32p^2-27p^4)} .$$

For the distribution of E for values of p from 0 to 1, see figure 1. The formula is about 90 percent efficiency throughout the repulsion phase, but compares badly with the Maximum Likelihood equation for high coupling. Nowhere does it give complete efficiency.

The expected frequencies may be found by substituting the most likely value of θ in the probabilities and multiplying by $n = 3496$.

TABLE 2

Comparison of observation with expectation, obtained by two different solutions (BRUNSON'S *data*).

		CRP_{θ_1}	CRp_{θ_1}	$(Cr+cR+c\sigma)P_{\theta_1}$	$(Cr+cR+c\sigma)p_{\theta_1}$	n
Observed		1907	300	1053	686	3946
Expected	*M. L.*	*1916.42*	*303.20*	*1043.08*	*683.30*	*3946.00*
	BR.	*1915*	*305*	*1044*	*682*	*3946*

χ^2 from Maximum Likelihood expectation $= 0.185$.
χ^2 from Brunson's expectation $= 0.2165$.
In each case there are 2 degrees of freedom from which to determine P.[1]

[1] FISHER (1922, 1923 and 1924) has shown that when a population, with which a sample is to be compared, has itself been reconstructed from the sample, the distribution of χ^2 is not known simply from the number of frequency classes. When using ELDERTON'S tables, the table must be entered with n' equal to *one more than the number of degrees of freedom* in which the sample may

Any number of formulae for the estimation of p may be invented. See FISHER (1928, in press).

The amount of information per plant obtained from the F_2 and its corresponding backcross may be compared with the amount of information obtainable from a simple backcross, that is, one in which complete classification is possible.

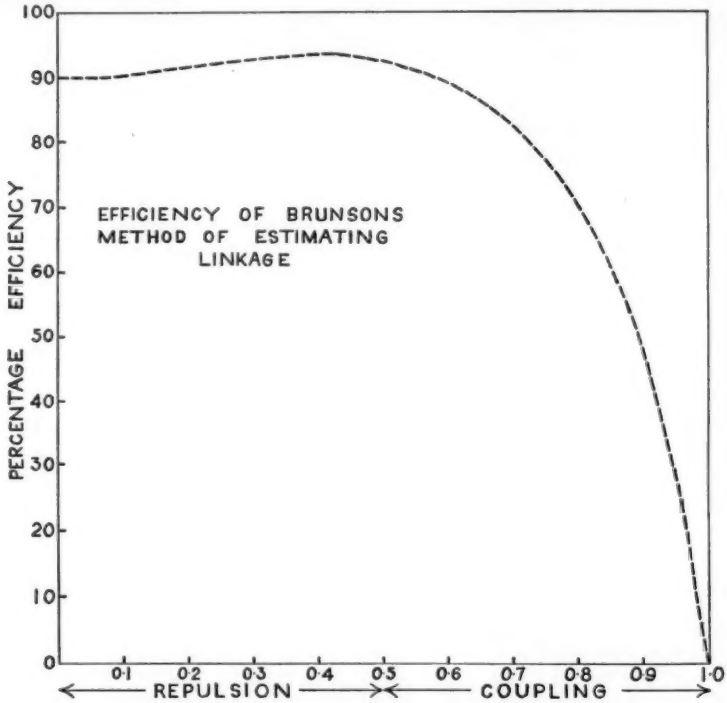

FIGURE 1.—Efficiency of BRUNSON's method of estimating linkage in an F_2 involving a factor linked to one of two complementary factors.

For a simple backcross:

$$V(p)=\frac{p(1-p)}{n}.$$

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differ from the reconstructed population. Doctor FISHER (1928) gives a table of χ^2 which is more convenient for use than that given by ELDERTON, and in using FISHER's table, the table is entered with *n equal to the number of degrees of freedom* in which the sample may differ from the reconstructed population.

Therefore the amount of information concerning p available per plant is

$$\frac{I(p)}{n}=\frac{1}{p(1-p)}.$$

In a backcross between an F_1 of the type under consideration and its triple recessive, the probabilities of the four classes will be:

CRP_{g_1}	CRp_{g_1}	$(Cr+cR+cr)P_{g_1}$	$(Cr+cR+cr)p_{g_1}$
$\frac{p}{4}$	$\frac{1-p}{4}$	$\frac{2-p}{4}$	$\frac{1+p}{4}$

And the maximum likelihood value of p is that for which

$$\frac{a}{p}-\frac{b}{1-p}-\frac{c}{2-p}+\frac{d}{1+p}=0$$

and the variance of p

$$\frac{1}{V(p)}=\frac{n}{4}\left(\frac{1}{p}+\frac{1}{1-p}+\frac{1}{2-p}+\frac{1}{1+p}\right)$$

$$V(p)=\frac{2p(1-p)(2-p)(1+p)}{n(1+2p-2p^2)}.$$

Then the amount of information concerning p available per plant is the reciprocal of the variance, divided by n

$$\frac{I(p)}{n}=\frac{1+2p-2p^2}{2p(1-p)(2-p)(1+p)}.$$

The amount of information available per plant relative to that obtainable from a simple backcross is

$$\frac{1+2p-2p^2}{2p(1-p)(2-p)(1+p)}\div\frac{1}{p(1-p)}=\frac{1+2p-2p^2}{2(2-p)(1+p)}.$$

A similar procedure may be applied to the Maximum Likelihood solution for the F_2 and to BRUNSON'S formula. The amount of information concerning p made available per F_2 plant by the Maximum Likelihood equation is

$$\frac{I(p)}{n}=\frac{3p^2(5+2p^2-4p^4)}{(2+p^2)(1+p)(1-p)(2-p^2)(1+3p^2)}.$$

Dividing by $1/p(1-p)$ we get the amount of information per F_2 plant relative to that supplied by a simple backcross

$$\frac{3p^3(5+2p^2-4p^4)}{(2+p^2)(1+p)(2-p^2)(1+3p^2)}.$$

The amount of information concerning p made available per F_2 plant by BRUNSON'S formula is

$$\frac{I(p_B)}{n}=\frac{108p^2}{32+32p^2-27p^4}.$$

Dividing by $1/p(1-p)$, we get the amount of information per F_2 plant relative to that supplied by a simple backcross

$$\frac{108p^2(1-p)}{32+32p^2-27p^4}.$$

These amounts of information are plotted as percentages for values of p from 0 to 1 in figure 2. It will be seen that for values of p from 0.7 to 1.0—

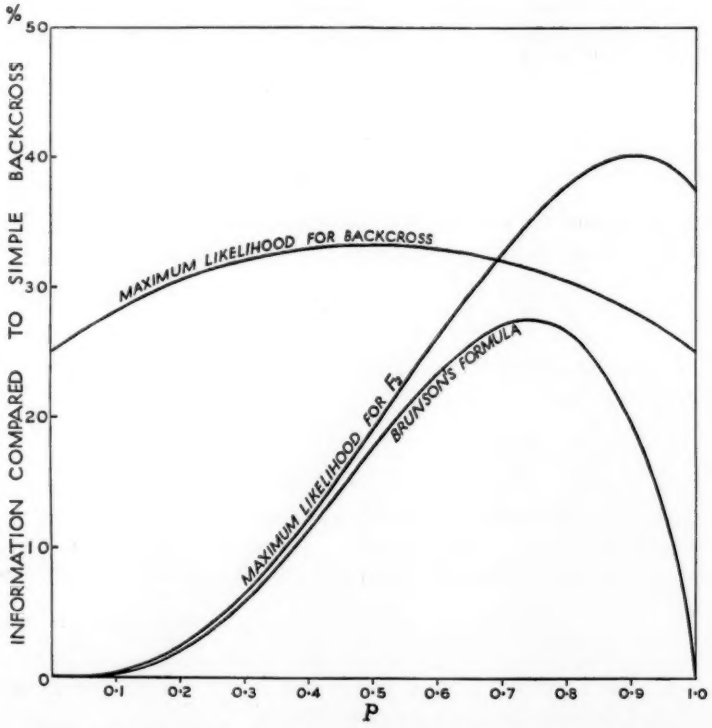

FIGURE 2.—A factor linked to one of two complementary factors: Amount of information concerning linkage supplied per plant by a backcross to a triple recessive, and by an F_2 using (a) Maximum Likelihood solution, (b) Brunson's solution. Amount of information per plant expressed as a percentage of that supplied by a simple backcross.

that is, 30 percent or less crossing over with coupling—an F_2 gives a better estimate of linkage than a backcross to the triple recessive. A simple backcross can, of course, be obtained by backcrossing to a double recessive, which is homozygous dominant for the independent complementary factor. In the present instance, backcrossing is impossible, because one of the linked factors is lethal in the recessive phase. In any case, it is not known which of the complementary factors is independent.

The amount of information supplied by the backcross is never more than 1/3 of what would be available if the classification could be completed. F_2s give very little information concerning linkage in the repulsion phase, and at best, in the coupling phase, give 40 percent of the information that would be supplied by a simple backcross of the same size.

A CASE INVOLVING DUPLICATE FACTORS

WOODWORTH (1921) has studied a case in soy beans in which one of two duplicate factors, *I* and *D*, for cotyledon colour is linked with a factor *V* for seed coat colour, and gives the figures given in table 3 for his F_2, after correcting his data to 15:1 for cotyledon colour. This correction is made necessary because seeds borne on the F_1 plants were classified for cotyledon colour, while seeds borne on F_2 plants were classified for seed coat colour.

TABLE 3

F_2 distribution for cotyledon colour (corrected to 15:1 nearest whole number) and seed coat colour (WOODWORTH'S *data*).

	ID+Id+iD	*id*	COAT COLOUR
V	150	14	164
v	64	0	64
Cotyledon colour	214	14	n=228

The probabilities of the four classes are given in table 4, where θ expresses the effect of the linkage, and, as before, is equal to p^2 if crossing over is the same in male and female.

TABLE 4

Probabilities of the four classes where θ expresses the linkage between either I or D, and V.

	ID+Id+iD	*id*	COAT COLOUR
V	$\frac{11+\theta}{16}$	$\frac{1-\theta}{16}$	$\frac{3}{4}$
v	$\frac{4-\theta}{16}$	$\frac{\theta}{16}$	$\frac{1}{4}$
Cotyledon colour	$\frac{15}{16}$	$\frac{1}{16}$	$n=1$

Then the most likely value of θ is that for which the first differential of the likelihood equation is 0,

$$\frac{150}{11+\theta}-\frac{64}{4-\theta}-\frac{14}{1-\theta}+\frac{0}{\theta}=0.$$

Then, clearly, the most likely value of θ is 0,—that is, complete linkage with repulsion.

The variance of this estimate is obtained, as before, by differentiating again, substituting the probabilities for the observed frequencies, and equating to $-1/V(\theta)$

$$\frac{1}{V(\theta)}=\frac{n}{16}\left\{\frac{1}{11+\theta}+\frac{1}{4-\theta}+\frac{1}{1-\theta}+\frac{1}{\theta}\right\}$$

$$V(\theta)=\frac{4(11+\theta)(4-\theta)(1-\theta)\theta}{n(11+2\theta-4\theta^2)}.$$

Then, since $\theta=p^2$ we can derive the variance of p as before.

$$V(p)=\frac{(11+p^2)(4-p^2)(1-p^2)}{n(11+2p^2-4p^4)}.$$

Substituting $\theta=0$ we get

$$V(p)=\frac{44}{11\times 228}=0.01754$$

$$\sigma(p)=0.132.$$

The F_2 evidence indicates, therefore, 0 percent crossing over, with a standard deviation of 13.2 percent. WOODWORTH (1921 and 1923) gives equations[2] for the estimation of linkage, which may be reduced to:

$$p_w^2=\theta_w=\frac{a+b-15c+d}{n}.$$

On applying this to the corrected data, WOODWORTH gets 13.25 percent crossing over. This, however, he shows to be due to the fact that the correction is to the nearest whole number, only, and when the exact corrected figures are taken, there is no evidence of crossing over.

The variance of θ_w may be obtained by applying equation 6 as before.

[2] WOODWORTH (1921) gives: $r=.25\sqrt{a+b+d+15c}$.
This is clearly an error for $r=.25\sqrt{a+b+d-15c}$.

$$S\left\{p\left(\frac{d\theta w}{da}\right)^2\right\}=\frac{11+\theta}{16}\times\frac{1}{n^2}+\frac{4-\theta}{16}\times\frac{1}{n^2}+\frac{1-\theta}{16}\times\frac{225}{n^2}+\frac{\theta}{16}\times\frac{1}{n^2}$$

$$=\frac{15-14\theta}{n^2}$$

$$\left(\frac{d\theta}{dn}\right)^2=\left(\frac{-a-b+15c-d}{n^2}\right)^2$$

$$=\left(\frac{-16n\theta}{16n^2}\right)^2$$

$$=\frac{\theta^2}{n^2}.$$

Then

$$\frac{1}{n}V(\theta_w)=\frac{15-14\theta-\theta^2}{n^2}$$

Then, since $\theta_w=p_w^2$, as before, the variance of p_w will be

$$V(p_w)=\frac{15-14p^2-p^4}{4np^2}.$$

Then, since $p=0$, in this case the variance of WOODWORTH'S estimate is infinitely large.

The expected numbers are obtained by substituting $\theta=0$ in the probabilities, and multiplying by $n=228$. Having corrected the ratio for cotyledon colour, and fitted an estimate of p, only 1 degree of freedom remains, and P =about 0.28. (See footnote to page 523.)

TABLE 5

Observed and expected frequencies for cotyledon colour and seed coat colour in soy beans. $\chi^2=1.1548$.

	$[ID+Id+iD]V$	$[ID+Id+iD]v$	idV	idv	n
Observed	150	64	14	0	228
Expected	*155.75*	*57.00*	*14.25*	*0.00*	*228*
Deviations	−6.75	+7.0	−0.25	0	

The probabilities of the classes in a backcross involving duplicate genes, one of which is linked to a third gene, will be

$$[ID+Id+iD]V:[ID+Id+iD]v:idV:idv$$

$$\frac{1+p}{4}\qquad\frac{2-p}{4}\qquad\frac{1-p}{4}\qquad\frac{p}{4}$$

and the variance of p will be

$$V(p)=\frac{2p(1-p)(2-p)(1+p)}{n(1+2p-2p^2)}$$

the same as for complementary factors.

The amounts of information concerning p available per plant, compared to a simple backcross are calculated as before and give

Maximum Likelihood equation for F_2

$$\frac{p(11+2p^2-4p^4)}{4(11+p^2)(4-p^2)(1+p)}$$

WOODWORTH'S formula for F_2

$$\frac{4p^2(1-p)}{15-14p^2-p^4}.$$

Maximum Likelihood equation for backcross

$$\frac{(1+2p-2p^2)}{2(2-p)(1+p)}.$$

These amounts of information are plotted as percentages of that obtainable from a simple backcross in figure 3. It is quite clear that very little information concerning linkage can be obtained from an F_2 involving duplicate factors under any circumstances, and such F_2s should always be carried on to F_3. A backcross to the triple recessive gives from 1/4 to 1/3 of the information per plant that would be obtained from a simple backcross.

TABLE 6

Soy bean F_2 segregating for seed coat colour and cotyledon colour: yellow cotyledon plants only included and these analysed according to F_3 behaviour (WOODWORTH'S *data*).

	TRUE BREEDING YELLOWS	SPLITTING YELLOWS		COAT COLOUR
		Double Het.	Single Het.	
V	57	45	45	147
v	55	3	5	63
Cotyledon colour	112	48	50	n=210

WOODWORTH is able to classify the 210 plants which came from embryos having yellow cotyledons into: (1) plants homozygous dominant for *I* or *D* or both; (2) plants segregating for both *I* and *D*; and (3) plants homozygous recessive for either *I* or *D* and segregating for either *D* or *I*. (See table 6.) Then the probabilities of the classes will be as given in table 7.

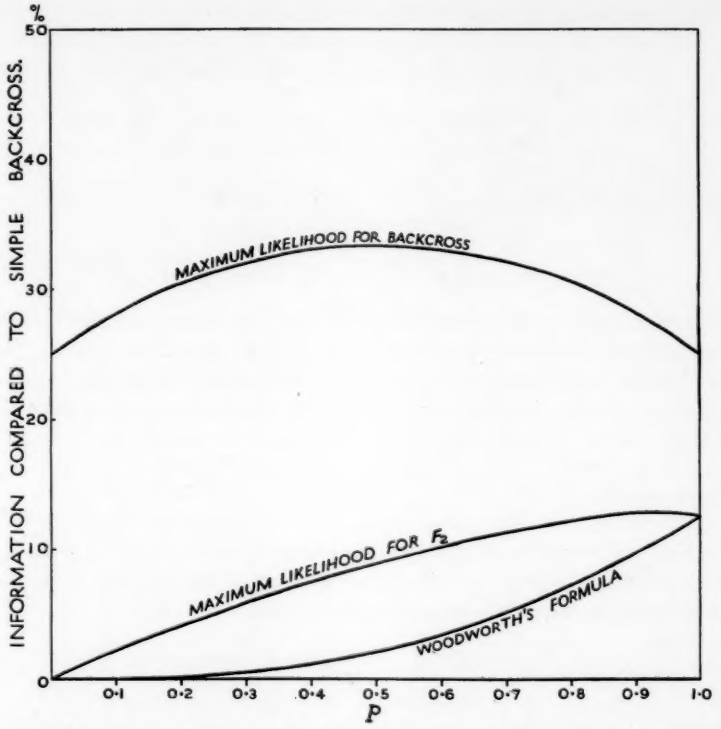

FIGURE 3.—A factor linked to one of two duplicate factors: Amount of information concerning linkage supplied per plant by a backcross to a triple recessive, and by an F_2, using (a) Maximum Likelihood solution, (b) Woodworth's solution. Amount of information per plant expressed as a percentage of that supplied by a simple backcross.

TABLE 7

Probabilities of the classes involved in the soy bean segregation tabulated in table 6.

	TRUE BREEDING YELLOWS	SPLITTING YELLOWS		COAT COLOUR
		Double Het.	Single Het.	
V	$\frac{3}{15}(1+2p-p^2)$	$\frac{4}{15}(1-p+p^2)$	$\frac{2}{15}(2-p)$	$\frac{11+p^2}{15}$
v	$\frac{1}{15}4-6p+3p^2)$	$\frac{4}{15}p(1-p)$	$\frac{2p}{15}$	$\frac{4-p^2}{15}$
Cotyledon colour	$\frac{7}{15}$	$\frac{4}{15}$	$\frac{4}{15}$	$n=1$

Then the garitholm of the likelihood will be

$$L=57\log\frac{3}{15}(1+2p-p^2)+45\log\frac{4}{15}(1-p+p^2)+45\log\frac{2}{15}(2-p)$$
$$+55\log\frac{4-6p+3p^2}{15}+3\log\frac{4p}{15}(1-p)+5\log\frac{2p}{15}\cdot$$

And the most likely value of p will be that for which

$$\frac{114(1-p)}{1+2p-p^2}-\frac{45(1-2p)}{1-p+p^2}-\frac{45}{2-p}-\frac{330(1-p)}{4-6p+3p^2}+\frac{8}{p}-\frac{3}{1-p}=x=0.$$

This equation may be multiplied up and solved by HORNER'S method, but an easier method is to obtain the value of p by successive trials. This method has the advantage that it avoids all algebraic manipulation, and provides not only an estimate of the linkage, but also an estimate of its standard error. A good guess for the first trial can usually be made after inspecting the data. In this example the F_2 indicated no crossing over, while the data in table 6 show that repulsion is not complete. A fair first trial is therefore $p=0.1$. Since the amount of information $[=1/V(p)]$ is the rate of change of x relative to p, the two final approximations, if worked out to sufficient accuracy, will give an estimate of the variance of p. The two final approximations used were $p=0.144$ and $p=0.115$.

When

$$p=0.114 \qquad x=+0.26904$$
$$p=0.115 \qquad x=-0.54503.$$

Therefore p is approximately 0.1143, or 11.43 percent crossing over. And for a change of 0.001 in p, x changes 0.81407, and the rate of change of $p=1/V(p)=814.07$

$$V(p)=0.001228$$
$$\sigma(p)=0.03504.$$

This may be checked by differentiating the likelihood equation a second time, equating to $-1/V(p)$ and substituting $p=0.11433$

$$\frac{1}{V(p)}=\frac{114(3-2p+p^2)}{(1+2p-p^2)^2}-\frac{45(1+2p-2p^2)}{(1-p+p^2)^2}+\frac{45}{(2-p)^2}+\frac{330(2-6p+3p^2)}{(4-6p+3p^2)^2}$$
$$+\frac{8}{p^2}+\frac{3}{(1-p)^2}=816.05$$

$$V(p)=0.001225$$
$$\sigma(p)=0.03500.$$

The two estimates of the variance differ by less than 0.5 percent.

We have, therefore, 11.43 percent crossing over with a standard deviation of 3.5 percent.

CONSIDERATION OF THEORY WHEN COMPLEMENTARY OR DUPLICATE FACTORS ARE THEMSELVES LINKED

These cases are rare, but may be considered for the sake of completeness.

(a) *Complementary Factors*

The probabilities of the two classes will be

$$\frac{2+\theta}{4}:\frac{2-\theta}{4}$$

where, as before, $\theta = p^2$ if crossing over is the same in male and female.

The most likely value of θ will be that for which

$$\frac{a}{2+\theta}-\frac{b}{2-\theta}=0$$

or

$$p^2=\theta=\frac{2(a-b)}{n}.$$

And

$$\frac{1}{V(\theta)}=\frac{n}{4}\left(\frac{1}{2+\theta}+\frac{1}{2-\theta}\right)$$

$$V(\theta)=\frac{(2+\theta)(2-\theta)}{n}.$$

And since $\theta = p^2$, we calculate the variance of p as before

$$V(p)=\frac{(2+p^2)(2-p^2)}{4p^2n}.$$

And the amount of information per plant compared to a (theoretical) simple backcross will be the reciprocal of this divided by $n/p(1-p)$

$$\frac{4p^3(1-p)}{4-p^4}$$

(b) *Duplicate Factors*

The probabilities of the two classes will be

$$\frac{4-\theta}{4}:\frac{\theta}{4}.$$

The most likely value of θ will be that for which

$$\frac{b}{\theta}-\frac{a}{4-\theta}=0 \quad .$$

or

$$p^2=\theta=\frac{4b}{n} \cdot$$

And differentiating again, substituting probabilities for a and b and equating to $-1/V(\theta)$ we get

$$\frac{1}{V(\theta)}=\frac{n}{4}\left\{\frac{1}{\theta}+\frac{1}{4-\theta}\right\}$$

$$V(\theta)=\frac{\theta(4-\theta)}{n}$$

$$V(p)=\frac{4-p^2}{4n} \cdot$$

And the amount of information per plant compared with a simple backcross will be

$$\frac{4p(1-p)}{4-p^2} \cdot$$

The probabilities of the classes in the two backcrosses will be

Complementary $\quad \frac{p}{2}[AB]:\frac{2-p}{2}[Ab+aB+ab]$

Duplicate $\quad \frac{2-p}{2}[AB+Ab+aB]:\frac{p}{2}ab.$

And the most likely values of p will be of those for which

$$\frac{a}{p}-\frac{b}{2-p}=0 \text{ for Complementary Factors.}$$

and

$$\frac{b}{p}-\frac{a}{2-p}=0 \text{ for Duplicate Factors.}$$

The variance of p is the same in each case:

$$\frac{1}{V(p)}=\frac{n}{2}\left(\frac{1}{p}+\frac{1}{2-p}\right)$$

$$=\frac{n}{p(2-p)}$$

$$V(p)=\frac{p(2-p)}{n}$$

Then the amount of information per plant relative to a simple backcross will be the reciprocal of the variance divided by $n/p(1-p)$

$$=\frac{1-p}{2-p}.$$

These amounts of information are plotted as percentages of the amount of information supplied by a simple backcross for values of p from 0 to 1. See figure 4.

Very little information concerning linkage can be obtained from an F_2 involving linked complementary factors. Where there is a considerable

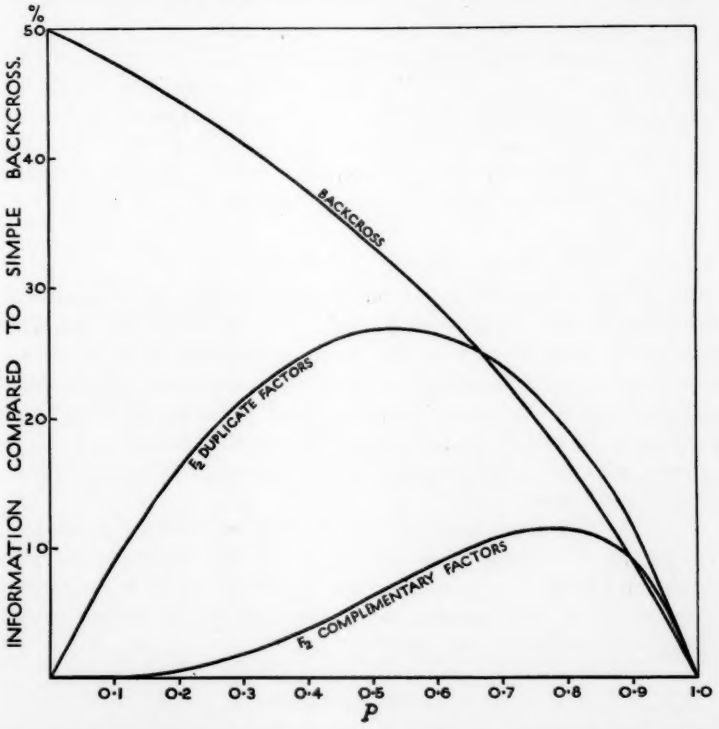

FIGURE 4.—Complementary factors themselves linked, and duplicate factors themselves linked. Amount of information concerning linkage supplied per plant by a backcross to a double recessive, and by F_2 using Maximum Likelihood solution. Amount of information per plant expressed as a percentage of that supplied by a simple backcross.

amount of crossing over—that is, where p is near 0.5—an F_2 involving linked duplicate factors will give about 1/4 of the information that would be given by a simple backcross of the same size. The backcrosses give up to 1/2 of the information obtainable from a simple backcross with high linkage in the repulsion phase, but become less efficient than the corresponding F_2s with high coupling.

SUMMARY

1. The "Method of Maximum Likelihood" developed by DOCTOR R. A. FISHER is applied to the problem of estimating linkage in cases involving complementary and duplicate factors.

2. Variances are calculated for existing formulae, and their efficiencies are determined to show that the "Method of Maximum Likelihood" is in all cases superior to any other method of estimation.

3. The amount of information supplied per plant by Maximum Likelihood formulae for F_2s and backcrosses, and by other formulae for F_2s is calculated and compared with the amount of information supplied per plant by a simple—that is, completely classified—backcross. (See figures 2, 3 and 4.) From these curves it is possible to estimate the size of family necessary to give any required degree of accuracy.

ACKNOWLEDGMENTS

The investigation here presented was carried out at ROTHAMSTED EXPERIMENTAL STATION under the guidance of DOCTOR R. A. FISHER, during a period of study leave from the Genetics Department of the COTTON RESEARCH STATION, Trinidad, B. W. I. The author is indebted to the EMPIRE COTTON GROWING CORPORATION for granting the leave, and to DOCTOR R. A. FISHER for suggesting the problem, and for the care and patience with which he supervised both its general development, and its detailed application.

Since the above was written a paper on the calculation of linkage intensities by F. V. OWEN has come under notice. FISHER'S Method of Maximum Likelihood is considered, but its particular virtue, namely, that, in the theory of large samples, no statistic can have a smaller sampling variance, is overlooked; see FISHER (1928).

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POLLEN-TUBE GROWTH IN CROSSES BETWEEN BALANCED CHROMOSOMAL TYPES OF *DATURA STRAMONIUM*[1]

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In an investigation on tetraploid plants of *Datura Stramonium* (BLAKESLEE, BELLING AND FARNHAM 1923) it was shown that the cross with diploids ($2n \times 4n$) is not successful. Over 212 attempts between various strains of $2n$ plants pollinated with pollen from $4n$ plants failed to set seed, as well as many more attempts made later. The reciprocal cross ($4n \times 2n$) produces some seeds, and this combination has given rise to triploid ($3n$) plants, but even this cross is difficult to accomplish and, of the few seeds obtained, only a small proportion have been brought to germination.

[1] One of a series of cooperative investigations made possible by grants from the AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE (1922–23) and from the JOSEPH HENRY FUND, NATIONAL ACADEMY OF SCIENCES (1924–26).

The present investigation on pollen-tube growth was undertaken in an effort to find an explanation for the difficulties encountered in making this cross. We are also including tests of pollen-tube growth in combinations of crosses between the balanced chromosomal types, $4n$, $3n$, $2n$, and n plants. These were tested with their own pollen and that of other members of this balanced series, all of them belonging to Line 1A, except a few of the tests made in the years previous to 1927, which belonged either to Line 1 or Line 1A.

The plants of Line 1 were seed-grown from a strain of purple-flowered armed, many noded plants originally obtained from Washington, D. C. This line had been rendered homozygous through many generations of inbreeding. The plants of Line 1A which were used in these tests represent a strain developed from a single haploid plant of Line 1.

Two unimportant combinations employing the pollen of $3n$ plants ($4n \times 3n$ and $n \times 3n$) were omitted. The former was made in previous years and shows the same general conditions as $2n \times 3n$ or $3n \times 3n$. The cross $n \times 3n$ was not tested. The general procedure and technique employed was similar to that of our other investigations on pollen-tube growth (BUCHHOLZ and BLAKESLEE 1928b, c) in which cut flowers were pollinated and kept for a given period of time (usually 12 hours) under conditions of constant temperature.

From microscopic slides prepared by dissection of the styles killed after known time intervals the following diagrams and statistical tables were prepared. Photographic reproductions of several such preparations are shown in figures 25–29. In the diagrams, the pollen tubes are represented as growing from the stigma toward the right. Only the forward ends of pollen tubes were counted and are plotted in the appropriate space (3 mm intervals), those whose ends appeared normal (see figure 28) are plotted above the datum line, those whose ends appeared swollen or abnormal due to bursting (see figure 25) are plotted below the datum line. Pollen grains which remained ungerminated on the stigma together with those which burst on the stigma during germination, two classes not always separated with certainty in making the counts, are plotted together, and, for the sake of simplicity, in the form of a single vertical bar at the left, half above and half below the datum line. These were actually observed scattered over the curved surface of the stigma through several space intervals. The counting was greatly facilitated by the use of two hand tally counters and a microscope equipped with a graduated mechanical stage with vernier, and an ocular with cross hairs. The field

width between cross hairs was .33 mm and nine of these transects were combined in the diagrams to represent 3 mm intervals.

In figures 25 to 28 are shown $2n$ styles with pollen tubes from $2n$ and $4n$ parents. Figure 25, with pollen from $4n$, shows the region just below the expanded stigma (6–13 mm from top of stigma) and figure 27 shows the similar region with pollen from $2n$. Figure 26 with pollen from $4n$, and figure 28 with pollen from $2n$, show the regions near the point reached by the longest pollen tube. Figures 25 and 26 are photographs from the same preparation as the diagram of figure 8 ($2n \times 4n$) and figures 27 and 28 are from a test similar to that shown in figure 2 ($2n \times 2n$).

The diagrams, figures 1 to 24, illustrate in detail the distribution of the pollen tubes in representative slides from the various tests. These and all other similar counts not represented by diagrams are given in table 2. In the tables the germinated portion of the gametophytes are grouped into two kinds, normal-appearing A and B, and abnormal-appearing C and D. Group A represents all pollen tubes which have grown to a point half as long as the longest normal pollen tube or longer, Group B are laggards or those which have grown less than half of this distance. Likewise, Group C represents burst or abnormal pollen tubes which have grown half, or more than half, of the distance of the longest normal pollen tube, and D represents the abnormal group which have grown less than half of this distance. Thus, if our diagrams, figures 1, 2, 3, and so forth were each divided by a vertical line at the mid-point, there would be four quadrants, two above and two below the datum line, lettered and related thus:

$$\left|\begin{matrix} BA \\ DC. \end{matrix}\right.$$

In table 2 both the actual numbers, and also the percentages, are given for individual tests. The percentages given in the first few columns are based on the total number of pollen grains accounted for in the test, while the percentages in the columns under Groups A, B, C, and D are based on the pollen tubes alone, not including the pollen remaining ungerminated on the stigma. Thus the pollen of a tetraploid plant on its own stigma usually shows about 24 percent ungerminated, and the percentage distribution of the A, B, C and D groups is based only on the 76 percent which germinated.

For convenience, we have prepared table 1 which shows only the general averages in percentage of each group of tests in table 2. Since table 1 does not include an equal number of tests of each type of cross, we are giving the total number of pollen grains accounted for (total gametophyte population) and also the number of styles whose counts were included in

each type of cross, in order that these may serve roughly as a measure of the statistical value of the data on which the percentages are based. The diagrams are given as representative examples showing the more exact distribution of the pollen tubes.

Not all of the pollinations were obtained in 1927. Some were made in previous years (but all since 1921) and under slightly different conditions, as indicated in the diagrams.

POLLEN-TUBE GROWTH IN DIPLOID PLANTS

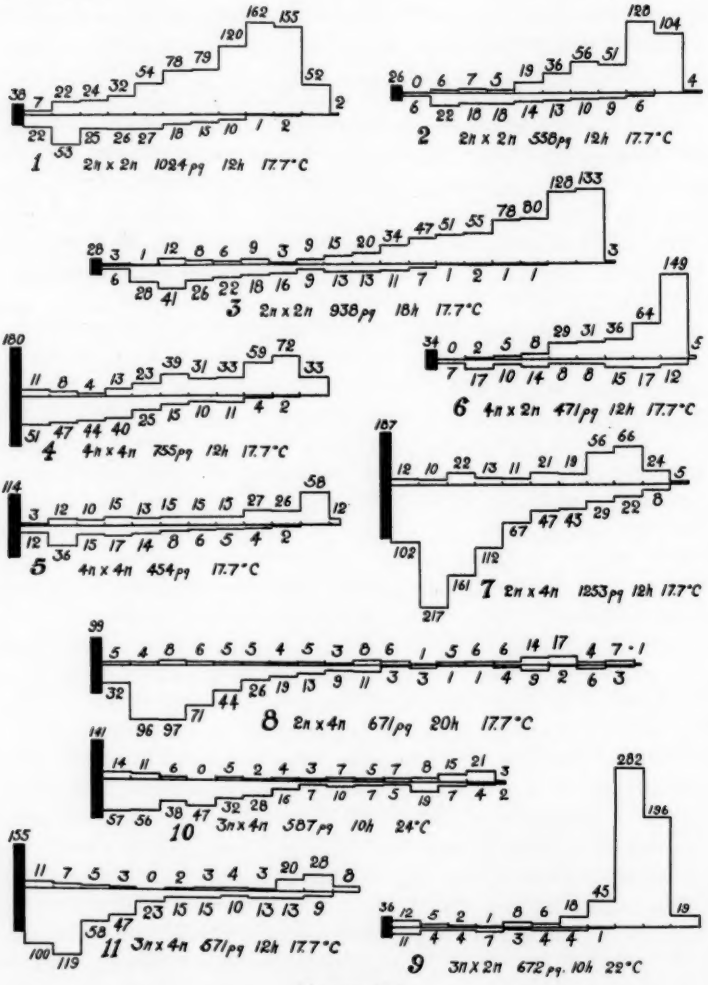
Figures 1 and 2 represent the curves of distribution of the ends of the pollen tubes in the styles of normal diploid plants after 12 hours at 17.7° C. Figure 3 belongs to the same set of tests, but was left growing for 18 hours. Self pollinations were the same as sib-crosses. The proportion of ungerminated pollen grains remaining on the stigma was small, being only about 4–5 percent. Their number is given and represented in the diagrams by the heavy vertical bar at the left.

These diploids give us distribution curves which serve as our standards of comparison. It will be seen in the diagrams and in figure 28 that the mode of frequency of the ends of normal-appearing pollen tubes was near the advancing front. The pollen tubes lagging behind the mode may represent to some extent individuals which were slow in germination due to imperfect contact with the stigma or retarded individuals due to crowding, but the abnormal pollen tubes, those below the datum line, and some of the slow-growing normal tubes may have been due to differences in nuclear or cytoplasmic constitution.

The mode of distribution of the abnormal pollen tubes was in the region near the stigma as indicated very accurately by the diagrams. It is also shown in the tables by the proportions in Groups C and D. The abnormal pollen tubes were present in small proportion in the pistils of all normal diploid plants, whether from self pollinations or sib crosses (see figure 27). They are found in the purest inbred strains. Their proportion is not always quite the same, and their abundance seems to bear some relation to the number of ungerminated pollen grains, and also to the number of retarded or slow-growing pollen tubes in the normal group represented above the datum line (Group B of tables). Thus, if there are few burst tubes, there is very often a greater proportion of retarded pollen tubes or ungerminated pollen grains.

The burst or abnormal pollen tubes (Groups C and D) may have been due in part to chromosomal irregularities from non-disjunction in microsporogenesis. They resemble the types of abnormal tubes which we have

found present in great abundance among the $(2n+1)$ chromosomal types which we have been investigating by the same methods. However, BELLING (BELLING and BLAKESLEE 1924b) observed only 0.4 percent non-disjunction in $2n$ plants, so that in the main they may be due to other causes.



FIGURES 1–11.

A number of swollen pollen tubes were found and these were counted as abnormals along with those which had burst. These swollen tubes may continue to grow slowly but they usually burst sooner or later. The burst pollen tubes have ceased growing and cannot reach the ovary for fertilization. They are an element of the pollen tube population which has been eliminated.

Figure 1 represents a pistil with 1024 pollen grains accounted for, and figure 2 with 558 has only about half as many. The difference between these two tests which were made at the same time shows the effect of crowding. In figure 1, the shape of the curve shows that the proportion of slower-growing pollen tubes among the normals was greater than it was in figure 2. This is also shown in the tables, where group B is 18 percent in figure 1 and only 6 percent in figure 2. However, figure 3 which is a large population (928 pollen grains applied at the same time) clearly shows a large proportion of retarded pollen tubes by the shape of the curve, but it does not show a large B group in the tables, where this value is only 6 percent. This is explained by the fact that in this 18-hour test these retarded tubes had moved forward; the diagram shows that the main body of these had just passed the mid point. Other conditions enter into this matter of crowding, as for example the size of the stigma and style which varies considerably. The number of burst or swollen tubes found also varies, so that these crowding effects are not always clearly shown and no effort was made in these tests to bring out extreme differences due to crowding.

It is obvious by comparing figure 3 with figure 1 that the mode of distribution of the abnormal group had moved forward very little, the difference being due to some of the swollen tubes included in the counts which continued to grow slowly during the extra 6-hour interval. There was also an increase in the proportion of the abnormal pollen tubes, due to additional bursting during the longer time interval.

Some of the burst pollen tubes were probably due to injuries received in manipulation, before killing and fixing was complete. These induced injuries were more or less distinguishable as such. They are found chiefly in the region near the mode of distribution of the ends of normal pollen tubes, where the latter are most numerous at the time of killing. We have checked against this experimental error by dissection of living styles, and also by rapid killing with a minimum of handling, and find that on the whole the error from this source is very nearly negligible. This condition may be reflected in some instances as shown in figure 6, where there were

more burst tubes than we might expect in the region of the mode of frequency of normal tubes.

POLLEN-TUBE GROWTH IN TETRAPLOID PLANTS

Figures 4 and 5 represent typical pollen-tube distribution curves in seed-grown Line 1A tetraploid plants ($4n \times 4n$), after 12 hours, at 17.7° C. Both the proportion of burst pollen tubes and the proportion of ungerminated pollen grains were much higher than in diploid plants. The ungerminated pollen was about 24 percent and this count included pollen grains burst on the stigma during germination.

As in diploids, the normal-appearing portion of the pollen-tube population (those above the datum line) are grouped into a distinct mode near the advancing front. The tables as well as the diagrams show that in comparison with $2n$ plants, there are more slow-growing normal pollen tubes in proportion to the number found grouped in the mode (figures 1 and 2). Figure 4 shows proportionately fewer lagging normal pollen tubes (13 percent in group B) than figure 5 (17 percent in Group B), but the effect of the greater crowding in figure 4 may have been to cause a greater proportion of bursting among these slower-growing pollen tubes. This is confirmed by the relative proportions found in the D groups.

The burst and swollen tubes were crowded mostly into a second mode located in the region 3–15 mm from the top of the stigma. They are much more conspicuous objects than normal tubes, and frequently a number of pollen tubes terminate in the same mass of extruded protoplasm, indicating that interference may have taken place.

The great abundance of burst pollen tubes may be explained in part by the proportionately high non-disjunction in the microsporogenesis of tetraploids, which amounts to about 30 percent (BELLING and BLAKESLEE 1924a).

We may estimate the excess of abnormal pollen tubes and ungerminated pollen grains of tetraploids over those of diploids from the data in table 1. This excess represents a difference of about 32 percent. Thus the present tests of pollen-tube growth show that 100 pollen grains from tetraploids yield about 32 more individuals which show abnormal performance or fail to germinate entirely, than this number of pollen grains from diploids. This is in close agreement with BELLING'S estimate of the total non-disjunction.

It should be pointed out in this connection that chromosomal irregularities such as a high non-disjunction is also indicated in the megasporogenesis of tetraploid plants. This condition is reflected in the number of

very small aborted ovules found on the enlarged region of the placenta among the seeds which may be observed to the best advantage in immature seed capsules. The percentage of unenlarged or aborted ovules in tetraploids is always high and varies between 35 and 50 percent, while in diploids this value is usually between 6 and 9 percent.

TETRAPLOID × DIPLOID CROSSES

In the cross $4n \times 2n$, pollen-tube growth is nearly the same as when diploids are self-pollinated. A number of such tests were made in different seasons, and figure 6, a 12-hour test at 17.7°C, will serve to illustrate these. Figure 17 shows another test in which Group A is greater, even though the population is larger. Though neither of these represent conditions of great crowding (the stigma and style of tetraploid plants are quite large), the spread of the A group is slightly greater in figure 17 than in figure 6. The more pronounced peak in the curve of figure 6 may be due to less crowding, but both tests seem to show favorable conditions of pollen-tube growth.

There is, obviously, no possibility that unfavorable pollen-tube growth might be responsible for the low fecundity of the cross $4n \times 2n$. The plants coming from this cross are chiefly $3n$ and $2n$, the latter resulting presumably from parthenogenesis of $2n$ female gametes. The number of seeds obtained from this cross is very low in proportion to the number of pollen tubes which appear to be available for fertilization. Thus in 1920–21 (BELLING and BLAKESLEE 1922, table 2) 41 capsules were obtained from 62 attempts, $4n \times 2n$. These 41 capsules contained 167 seeds, an average of 2.6 seeds per capsule.

We may safely assume that at least 500 pollen tubes may enter the ovary in the pollinations $4n \times 2n$. In estimating the seed to pollen-tube ratio, therefore, we can calculate 1 seed per 192 pollen tubes reaching the ovary. Furthermore, only 16 seedlings were obtained from 167 seeds, which gives a ratio of one seedling per 1900 pollen tubes reaching the ovary. The number of seedlings which are actually triploid plants, and therefore the result of fertilization, is still lower (one $3n$ plant per 13000 pollen tubes reaching the ovary). This ratio would be widened still further if records of all attempts at pollinations were included, rather than data from seed-bearing capsules only.

There are always many aborted ovules and small seeds obtained in these crosses, $4n \times 2n$, even though the seed capsules contain only a few viable seeds. Some of these ovules have enlarged considerably and may be classified from external appearance as small seeds. There are very many

others which enlarged only slightly. Still others too microscopic to be included in seed counts fail to enlarge at all, and correspond in size to the abortive ovules observable scattered over the placental surface in immature capsules of tetraploid plants.

From this, it is obvious that the relative infertility of the cross $4n \times 2n$ is due to causes other than pollen-tube growth, such as failure of proper union of the sperms with both egg and polar nucleus, failure of the zygotes to develop after fertilization, or nutritional difficulties resulting in death of the embryo during seed development. Combined with this early abortion we have poor seed germination.

DIPLOID × TETRAPLOID CROSSES

In the cross $2n \times 4n$, a distinctly different picture of pollen-tube growth is obtained (see figures 25 and 26). We have shown in a previous publication (BUCHHOLZ and BLAKESLEE 1927b) that the distribution curves for pollen tubes of this cross have a backward skew. If a separation is made of the abnormal and normal-appearing pollen tubes, we obtain distribution curves similar to the one shown in figure 7, which shows the results of a 12-hour test at 17.7°C. The pollen of the $4n$ plant actually germinates better on the diploid stigma than it does on the $4n$ stigma; on $2n$, 85 percent germinate; on $4n$, 76 percent germinate. All of our tests of this cross made at various times during the past few years show conditions very similar to these.

The proportion of abnormal pollen tubes is very large. Figure 7 shows a condition in which only 21 percent of the total pollen-tube population remained normal-appearing after 12 hours. This is one of the most favorable of our tests. Among the germinated portion of the gametophyte population, the A group varied from 10–18 percent as shown in table 2 with an average of only 14 percent.

Figure 8 shows one of these styles with a population of 671. This test was made at the same time as the one used for figure 7 but the pollen tubes were kept growing for 20 hours. Here the normal-appearing pollen tubes comprise only 18 percent, while the abnormal or burst portion represents 67 percent of the total population. The A group is only 12 percent (table 2) and at this stage bursting is still taking place. Thus, there is little probability that more than a few scattered individuals of the remaining pollen tubes would have reached the ovary. These tests were conducted under conditions favorable to pollen-tube growth (BUCHHOLZ and BLAKESLEE 1927b, c). In the field or greenhouse at variable high temperatures, the conditions are less favorable.

Even if an occasional pollen tube should reach the ovules, the chances of obtaining seeds are, as we have shown in the reciprocal cross, only about 1 in 192, and the chances for the survival of seedlings are not over one in 1900. The sterility of the cross $2n \times 4n$ is, therefore, due primarily to difficulties in pollen-tube growth.

TRIPLOID × DIPLOID CROSSES

The cross $3n \times 2n$ has been successfully made many times. It is from this cross that the primary $(2n+1)$ chromosomal types have been obtained repeatedly in our cultures. In a recent publication (BLAKESLEE 1927) 936 such offspring are tabulated, showing aside from 270 normal plants, 498 $(2n+1)$ primaries, and 310 double $(2n+1+1)$ forms with 13 unidentified plants.

Pollen-tube growth is very favorable in this cross. Figure 9 represents such a case and this is from a 10-hour test made in a previous year, kept at 22°C. The triploid plants used in these tests for styles as well as pollen parents were scions from a grafted Line 1A plant and were therefore derived from the same plant used in other breeding experiments. Since we knew that this cross gives favorable pollen-tube growth under various conditions, we did not repeat these tests in 1927.

The distribution of the pollen tubes of figure 9 with the small proportion of burst pollen tubes found, indicates conditions of very favorable pollen-tube growth. Tables 1 and 2 show that the A group comprises 88 percent of the pollen-tube population which is a much higher figure than that in any other combination which we have tested. There are fewer laggard pollen tubes than in $2n \times 2n$ crosses, but this may be partly explained by the fact that the styles of triploids are considerably larger than those of diploid plants so that crowding is lessened.

TRIPLOID × TETRAPLOID CROSSES

Figures 10 and 11 show the distribution of the pollen tubes in a test of the cross $3n \times 4n$. Three such tests were made in 1927, 12 hours at 17.7°C., and two were made in previous years, 10 hours at 24°C. All five tests are included in the tabulation, and are consistent in showing unfavorable pollen-tube growth. An average of 27.6 percent of the pollen fails to germinate, in comparison with 24 percent in the cross $4n \times 4n$, and 14.6 percent in the cross $2n \times 4n$. If we consider the germinated portion of the gametophyte population, we will note that Group D includes 62 percent, Group C 14 percent, Group B 9 percent and Group A 15 percent (averages, table 1). Except for the differences in the ungerminated portion

TABLE 1

Summary for each combination of tests.

TYPE OF CROSS		NUMBER OF TESTS	GRAND TOTAL (Gametophytes recorded)	UNGERMINATED BURST POLLEN ON STIGMA		POLLEN TUBES SWOLLEN OR BURST WITHIN STYLE		POLLEN TUBES WITHIN STYLE APPEARING NORMAL		TOTAL NUMBER OF TUBES	POLLEN TUBES SWOLLEN OR BURST IN REGION CORRESPONDING TO GROUP B Group D		POLLEN TUBES BURST AFTER GROWING LONGER THAN GROUP B Group C		RETARDED NORMAL POLLEN TUBES Group B		FORWARD GROUP OF NORMAL POLLEN TUBES Group A		GROUP A, PERCENT BASED ON GRAND TOTAL
				Number	Percent	Number	Percent	Number	Percent		Number	Percent	Number	Percent	Number	Percent	Number	Percent	
1*n*×1*n*	1923	2	396	123	31.0	100	25.3	173	43.7	273	61	2.41	39	14.3	79	28.9	94	34.4	23.7
1*n*×2*n*	1924	1	264	15	5.7	22	8.3	227	86.0	249	16	6.4	6	2.4	24	9.6	203	81.5	76.8
1*n*×4*n*	1924	1	557	81	14.5	378	67.9	98	17.6	476	266	55.9	112	23.5	52	10.9	46	9.7	8.3
	1927	1																	
2*n*×1*n*	1927	3	2039	608	29.8	138	6.8	1293	63.4	1431	84	5.9	54	3.8	243	17.0	1050	73.3	51.5
2n×2n	1927	3	**3514**	**145**	**4.1**	**639**	**18.2**	**2730**	**77.7**	**3369**	**501**	**14.9**	**138**	**4.1**	**421**	**12.2**	**2309**	**68.8**	**65.7**
	1926	2																	
2*n*×3*n*	1927	2	1079	984	91.2	55	5.1	40	3.7	95	47	49.5	8	8.4	26	27.4	14	14.7	1.2
2*n*×4*n*	1927	4	3380	498	14.7	2238	66.2	644	19.0	2882	1963	68.2	275	9.5	196	6.8	448	15.5	13.3
3*n*×1*n*	1923	1	542	212	39.1	42	7.7	288	53.2	330	25	7.6	17	5.2	49	14.8	239	72.4	44.1
3*n*×2*n*	1923	1	672	36	5.4	42	6.2	594	88.4	636	30	4.7	12	1.9	32	5.0	562	88.4	83.6
3*n*×3*n*	1923	1	1120	1009	90.1	67	6.0	44	3.9	111	66	59.4	1	0.9	25	22.5	19	17.1	1.7
	1927	1																	
3*n*×4*n*	1925	2	2404	660	27.6	1343	55.8	397	16.5	1740	1127	64.7	216	12.4	148	8.5	249	14.3	10.4
	1927	3																	
4*n*×1*n*	1927	2	725	252	34.8	64	8.8	409	56.4	473	38	8.0	26	5.5	28	5.9	381	80.6	52.5
4*n*×2*n*	1927	2	1137	63	5.5	215	18.9	859	75.6	1074	118	11.0	97	9.0	82	7.6	777	72.4	67.4
4*n*×4*n*	1927	2	1209	294	24.2	368	30.4	547	45.3	915	316	34.5	52	5.7	134	14.6	413	45.2	34.2

of the pollen, these tests are very similar to the $2n \times 4n$ cross and indicate very unfavorable conditions of pollen-tube growth. It is possible that occasionally a few pollen tubes might reach the ovary, and if difficulties in fertilization or survival of zygotes are not as great as in the cross $4n \times 2n$ we might expect an occasional seed. Offspring from this cross, however, have not yet been obtained.

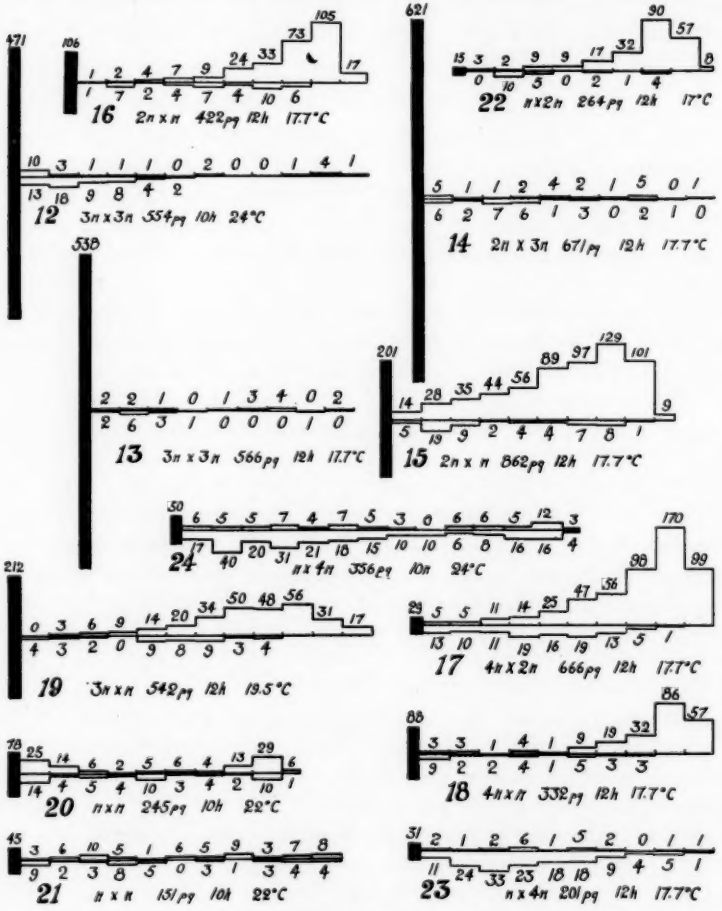
THE POLLEN OF TRIPLOIDS

The pollen of triploids contains a great range of types with variable chromosome numbers. There are a few grains with 12 chromosomes, which are the same as those produced by diploid plants, and a few with 24 chromosomes, similar to those from tetraploids, with a great preponderance of pollen grains having chromosome numbers between these two extremes.

After the formation of microspores, there is an important period of growth in the male gametophyte, before the pollen is actually matured as such. This is true of the pollen in all normal diploid plants as well as in triploids and tetraploids. The microspores of diploid plants have a volume of about 2900 cubic microns immediately after reduction and at their maturity the volume of the pollen grains is about 70,000 cubic microns, which represents a 24-fold increase in volume. In triploids during this stage of growth of the microspores, a considerable proportion, 43.6 percent (BLAKESLEE and CARTLEDGE 1926 and 1927) of the pollen becomes abortive. This abortive pollen probably represents a fraction containing the most unfavorable chromosomal combinations, which are unable to develop further as gametophytes and do not reach the stage of matured pollen grains.

Mature pollen grains in Datura have two nuclei, the tube nucleus and the generative nucleus; the former, a larger spheroidal body only slightly denser than the cytoplasm; the latter a much smaller, very dense rod-shaped structure, as observed in stained pollen grains from normal plants cleared in chloral hydrate. Before a pollen grain is matured from a microspore, it must not only enlarge greatly and become filled with reserve food substances, but its nucleus must undergo at least one division. Evidently not all of the pollen grains of a triploid plant are able to pass successfully through these stages of development and many microspores abort in various stages. An examination of this pollen shows evidence of a great range in the sizes of the shrunken aborted grains, as well as of the grains which survive to become rounded out and stain deeply. There is, therefore, a distinct phase of gametophytic selection here, and the inference is

obvious, that this elimination is to some extent differential, that the gametophytes with deficiencies and chromosomal combinations highly unfavorable to the continued growth of the gametophyte, are the ones



FIGURES 12–24.

eliminated early in various stages as aborted pollen, while the combinations more nearly normal for the gametophytes are the ones which increase their volume greatly and develop to maturity. Their mature size is prob-

ably in part proportional to their chromatin content, at least for the grains with 12, 24, and 36 chromosomes, in which the chromosome conditions are n, $2n$ or $3n$. In the great majority which have 12 plus several extra chromosomes, the sizes are probably also more or less conditioned by the combinations of extra chromosomes present.

POLLEN-TUBE GROWTH IN TRIPLOID PLANTS

Figure 12 shows the condition in a pollination $3n \times 3n$, in which there were 471 pollen grains ungerminated on the stigma, plus 83 which had germinated and produced pollen tubes. There were also many of the aborted grains (not plotted) which remained observable on the stigma. The 83 which had germinated represent only 15 percent of the pollen grains matured; 54 of them had burst within the region of the style near the stigma. Only 6 tubes, or possibly 8, may be considered by their performance as having 12 chromosomes, or a proportion of the total gametophytes between 1 and 1.5 percent. Figure 12 shows the results of a 10-hour test made prior to 1927 exposed at 24° C. Figure 13 is a 12-hour test made in 1927 at 17.7° C. and shows a much lower percentage of pollen germination on the stigma. Only 5 percent of the pollen grains germinated, against 15 percent in figure 12. The difference between these two tests is less pronounced when we consider only the normal-appearing pollen tubes (see also table 2) which are 3 percent in figure 13 against 5 percent in figure 12. If Group A and Group B are compared, we find that figure 12 has more in the B group while figure 13 has more in the A group. Basing the percentages in the A group on the total number of pollen grains accounted for, the percentages are very nearly equal; 1.6 percent for figure 12 and 1.7 percent for figure 13. The difference between the two tests is therefore one which may be attributed to the number of pollen grains with extra chromosomes which happened to germinate under the different conditions of the two tests, or to an experimental error through loss of ungerminated pollen during manipulation.

There is obviously very little crowding among the gametophytes which germinate and begin to grow within the style. Probably the pollen grains with a single extra chromosome may germinate, if the combinations are able to grow at all, but only the pollen tubes with 12 chromosomes will be likely to reach the ovary in most cases. The pollen of triploids cannot be crowded sufficiently on a stigma to provide more than a limited number of pollen tubes with 12 chromosomes and this, therefore, results in the fertilization of only a very few of the ovules in an ovary. Though fewer seeds are obtained from the selfing of triploid plants, $3n$ parents produce

seeds in considerable numbers when pollinated with the pollen of diploid plants. In another publication (BLAKESLEE 1927) an analysis of the progeny of triploid plants has been given.

DIPLOID × TRIPLOID CROSSES

The pollen of triploids, when germinated on the stigmas of diploid plants, gives very similar results to those just described. Figure 14 represents such a cross. The proportion of the pollen which may represent gametophytes with 12 chromosomes may be estimated from the data in table 2, by taking the number of pollen tubes in the A group and calculating their percentage of the total number of pollen grains accounted for under total gametophytes. In (27–211–1), this is 1.2 percent and in figure 14, this is 1.3 percent.

The expected random assortment of chromosomes in triploids has been confirmed by BELLING (BELLING and BLAKESLEE 1922). Such assortment should produce only 0.024 percent pollen grains with 12 chromosomes, the tubes from which should be found in our group A (compare tables). In this group of normal-appearing tubes, however, we have found about 1.5 percent of unaborted pollen, or about 0.85 percent of the total pollen population applied to the stigma. This is about 35 times the number expected. This figure would be decreased if any considerable number of pollen grains which had burst or remained ungerminated on the stigma were lost during manipulation.

From this fact we may conclude that in triploids some, as yet unknown, factors are responsible for the number of chromosomes in the male gametophytes offered to the processes of developmental selection. A somewhat similar situation is found in the female gametophytes of triploids (BELLING and BLAKESLEE 1922).

THE POLLEN OF HAPLOID PLANTS

Haploids produce two classes of pollen, pollen by "pseudo-reduction," in which the 12 chromosomes separate so that there are 6–6, 5-7, 4–8, and so forth, or always less than one complete set of 12 chromosomes, passing into the resulting micropsores (BELLING and BLAKESLEE 1926) and pollen by non-reduction, in which the number of chromosomes is 12. The pseudo-reduction microspores very generally abort, and there is quite a range in the sizes of the aborted grains, indicating that some of them may have enlarged before they aborted. BLAKESLEE and CARTLEDGE (1926, 1927) have estimated these aborted grains at 88 percent of the pollen count at the time when the pollen is mature, and have found that the conditions

under which the plants are growing result in slight differences in the proportion of "bad pollen". Grafted scions of the haploid plant 20745(4) were used in these tests on pollen-tube growth.

It is the remaining 12 percent of the pollen attributed primarily to non-reduction with which we are concerned in these tests of haploid pollen. It has been presumed that this pollen has 12 chromosomes. However, the different tests employing the pollen from haploids show that some differences exist, for we have a high proportion of this pollen (23 percent–42 percent) unable to germinate on the stigmas of $2n$ plants, or the stigma of any member of the balanced series.

DIPLOID × HAPLOID CROSSES

Since the functional pollen of haploids is scanty it was necessary to combine the pollen of several anthers in making these tests, in order that the number of fully matured grains applied to the stigma might be comparable to our $2n \times 2n$ tests. Our counts of the proportion of aborted pollen, made on the stigmas of several of the tests (data not included in the diagrams or tables) gave only 61–81 percent (average 76 percent) aborted pollen, but it is probable that some of the aborted grains were lost during the manipulation of mixing the pollen and pollinating the stigmas, and we are not certain that all abortive pollen grains present on the stigmas of our preparations are observable.

Figures 15 and 16 show the pollen-tube distribution curves of crosses $2n \times n$. Aside from the ungerminated pollen, these distribution curves are similar to those of $2n \times 2n$ tests and it is obvious that a very high proportion of these pollen tubes behave as if they had 12 chromosomes. These tests resemble diploid selfs and sib crosses even in respect to the details of the crowding effect. Figure 15, which has twice as many pollen tubes as figure 16, has a much greater proportion of retarded tubes (a larger B group, table 2) while another case (27–181–1, table 2) of intermediatesized population, shows the intermediate condition of distribution.

Some abnormal pollen tubes are found, resembling the abnormals of the $2n \times 2n$ tests but in much smaller proportion, less than half as many, which may be significant. It is possible that a chromosomal complex, which in a cytoplasm derived from a diploid would permit germination, prevents germination when the cytoplasm is derived from a haploid.

TETRAPLOID × HAPLOID CROSSES

Figure 18 shows a diagram of the pollen-tube distribution curve in a cross $4n \times n$. In this case, 26 percent of the matured pollen grains failed

TABLE 2

Pollen-tube distribution in individual styles.

TYPE OF CROSS AND IDENTIFICATION NUMBER	GRAND TOTAL (Gametophytes recorded)	UNGERMINATED POLLEN REMAINING ON STIGMA	POLLEN GRAINS BURST ON STIGMA	PERCENT UNGERMINATED AND BURST POLLEN	POLLEN TUBES APPEARING SWOLLEN OR BURST WITHIN STYLE		POLLEN TUBES APPEARING NORMAL		TOTAL NUMBER OF POLLEN TUBES	POLLEN TUBES SWOLLEN OR BURST IN REGION CORRESPONDING TO GROUP B		POLLEN TUBES BURST AFTER GROWING LONGER THAN GROUP B		RETARDED NORMAL POLLEN TUBES		FORWARD GROUP OF NORMAL POLLEN TUBES	
					Number	Percent	Number	Percent		Group D		Group C		Group B		Group A	
$n \times n$ (figure 20)	245	25	53	31.9	57	23.3	110	44.9	167	33	19.8	24	14.4	48	28.7	62	37.1
$n \times n$ (figure 21)	151	26	19	29.8	43	28.5	63	41.7	106	28	26.4	15	14.2	31	29.2	32	30.2
$n \times 2n$ (figure 22)	264	7	8	5.7	22	8.3	227	86.0	249	16	6.4	6	2.4	24	9.6	203	81.5
$n \times 4n$ (figure 23)	201	8	23	15.4	146	72.7	24	11.9	170	106	62.4	40	23.5	15	8.8	9	5.3
$n \times 4n$ (figure 24)	356	20	30	14.0	232	65.2	74	20.8	306	160	52.3	72	23.5	37	12.1	37	12.1
$2n \times n$ (figure 15)	862	50	151	23.3	59	6.8	602	69.9	661	38	5.7	21	3.2	158	23.9	444	67.2
$2n \times n$ (27–181–1)	755	130	171	39.9	38	4.9	416	55.2	454	26	5.7	12	2.6	65	14.3	351	77.4
$2n \times n$ (figure 16)	422	21	85	25.1	41	9.7	275	65.2	316	20	6.3	21	6.6	20	6.3	255	80.7
$2n \times 2n$ (figure 1)	1024	6	32	3.7	199	19.4	787	76.8	986	165	16.7	34	3.5	173	17.5	614	62.3
$2n \times 2n$ (figure 2)	558	6	20	4.7	116	20.7	416	74.6	532	73	13.7	43	8.1	34	6.4	382	71.8
$2n \times 2n$ (figure 3)	938	7	21	3.0	215	22.9	695	74.1	910	179	19.7	36	4.0	68	7.5	627	68.8
$2n \times 2n$ (26–3)	600	5	19	4.0	54	9.0	522	87.0	576	47	8.1	7	1.2	96	16.7	426	74.0
$2n \times 2n$ (26–4)	394	11	18	7.4	55	13.9	310	78.7	365	37	10.1	18	4.9	50	13.7	260	71.3
$2n \times 3n$ (figure 14)	671	497	124	92.5	28	4.2	22	3.3	50	22	44.0	6	12.0	13	26.0	9	18.0
$2n \times 3n$ (27–211–1)	408	279	84	89.0	27	6.6	18	4.4	45	25	55.6	2	4.4	13	28.9	5	11.1
$2n \times 4n$ (figure 7)	1253	38	149	14.9	807	64.4	259	20.7	1066	664	62.3	143	13.4	71	6.7	188	17.6
$2n \times 4n$ (27–154–2)	610	29	52	13.3	449	73.6	80	13.1	529	404	76.3	45	8.5	36	6.8	44	8.3
$2n \times 4n$ (27–160–2)	846	30	101	15.5	530	62.7	185	21.8	715	479	67.0	51	71.1	38	5.3	147	20.6
$2n \times 4n$ (figure 8)	671	19	80	14.8	452	67.3	120	17.9	572	416	72.7	36	6.3	51	8.9	69	12.1

TABLE 2 (Continued).

TYPE OF CROSS AND IDENTIFICATION NUMBER	GRAND TOTAL (Gametophytes recorded)	UNGERMINATED POLLEN REMAINING ON STIGMA	POLLEN GRAINS BURST ON STIGMA	PERCENT UNGERMINATED AND BURST POLLEN	POLLEN TUBES APPEARING SWOLLEN OR BURST WITHIN STYLE		POLLEN TUBES APPEARING NORMAL		TOTAL NUMBER OF POLLEN TUBES	POLLEN TUBES SWOLLEN OR BURST IN REGION CORRESPONDING TO GROUP B		POLLEN TUBES BURST AFTER GROWING LONGER THAN GROUP B		RETARDED NORMAL POLLEN TUBES		FORWARD GROUP OF NORMAL POLLEN TUBES	
					Number	Percent	Number	Percent		Group D		Group C		Group B		Group A	
$3n \times n$ (figure 19)	542	74	138	39.1	42	7.7	288	53.2	330	25	7.6	17	5.2	49	14.8	239	72.4
$3n \times 2n$ (figure 9)	672	5	31	5.4	42	6.2	594	88.4	636	30	4.7	12	1.9	32	5.0	562	88.4
$3n \times 3n$ (figure 13)	566	485	53	95.0	13	2.3	15	2.7	28	12	42.8	1	3.6	5	17.9	10	35.7
$3n \times 3n$ (figure 12)	554	315	156	85.1	54	9.7	29	5.2	83	54	65.0	0	0.0	20	24.1	9	10.9
$3n \times 4n$ (figure 11)	671	72	83	23.1	422	62.9	94	14.0	516	360	69.8	62	12.0	27	5.2	67	13.0
$3n \times 4n$ (27–196–2)	362	45	73	32.6	171	47.2	73	20.2	244	126	15.7	45	18.4	24	9.9	49	20.0
$3n \times 4n$ (27–196–3)	297	52	72	41.7	136	45.8	37	12.5	173	122	70.5	14	8.1	16	9.2	21	12.1
$3n \times 4n$ (figure 10)	587	53	88	24.0	335	57.0	111	18.9	446	276	61.9	59	13.2	43	9.6	68	15.3
$3n \times 4n$ (23–14)	487	58	64	25.9	279	57.3	82	16.8	361	243	67.3	36	10.0	38	10.5	44	12.2
$4n \times n$ (27–198–2)	393	77	87	41.7	35	8.9	194	49.4	229	20	8.7	15	6.6	16	7.0	178	77.7
$4n \times n$ (figure 18)	332	20	68	26.5	29	8.7	215	64.8	244	18	7.4	11	4.5	12	4.9	203	83.2
$4n \times 2n$ (figure 17)	666	7	22	4.3	107	16.1	530	79.6	637	67	10.5	40	6.3	55	8.6	475	74.6
$4n \times 2n$ (figure 6)	471	9	25	7.2	108	22.9	329	69.9	437	51	11.6	57	13.0	27	6.2	302	69.2
$4n \times 4n$ (figure 4)	755	48	132	23.8	249	33.0	326	43.2	575	218	37.9	31	5.4	77	13.4	249	43.3
$4n \times 4n$ (figure 5)	454	43	71	25.1	119	26.2	221	48.7	340	98	28.9	21	6.2	57	16.8	164	48.2

to germinate on the stigma. The germinated 74 percent were distributed within the style so that Group A contained 83 percent of them. By comparing this with Group A in the tables, it will be noted that this value is high, and indicates conditions of favorable pollen-tube growth. (Another case (27–198–2), not represented by a diagram shows 42 percent of the pollen ungerminated, but the distribution of the germinated portion is very similar to that in figure 18). Neither of these tests represents conditions of crowding, for the $4n$ pistil is large and there are few retarded pollen tubes. It is apparent that the cross $4n \times n$ is a possible combination from the standpoint of pollen-tube growth and compares very favorably with the cross $4n \times 2n$ (figure 17). A small proportion of abnormal pollen tubes produced by the pollen from a haploid is the rule in $4n \times n$ crosses.

TRIPLOID × HAPLOID CROSSES

Figure 19 shows the distribution of the pollen tubes in a $3n \times n$ cross in a 12 hour test made in a previous year at 19.5°C. This test was not repeated in 1927. It is similar in essentials to the crosses $4n \times n$ and $2n \times n$, and represents a combination with conditions of favorable pollen-tube growth. The ungerminated portion of the pollen remaining on the stigma is 39 percent and the grouping and distribution of the normal pollen tubes is rather spread and not entirely typical of other favorable combinations.

HAPLOID × HAPLOID CROSSES

Figures 20 and 21 show diagrams of the condition and distribution of the pollen tubes in $n \times n$ tests. Both of these were made previous to 1927. As in all other cases in which haploid pollen was tested a high proportion (30–32 percent) remained ungerminated on the stigma. Compared with the performance of this pollen when tested on styles of $2n$, $3n$ and $4n$ plants, the conditions of pollen-tube growth in $n \times n$ tests are less favorable, but they are not entirely unfavorable and much more satisfactory than the $2n \times 4n$ and $3n \times 4n$ crosses, as will be seen by a study of the proportions in the A and B groups in the tables.

The stigmas and styles of haploid plants are small and the number of pollen grains which could be applied to their stigmas by the same methods used in our other crosses was for this reason smaller. On the other hand, the styles are about 2/3–3/5 as long as those of $2n$ plants, and these short styles present a condition which tends to favor the survival of a sufficient number of normal pollen tubes in selfed haploid flowers to accomplish fertilization.

HAPLOID × DIPLOID CROSSES

The cross $n \times 2n$ was only attempted a few times and not repeated in 1927. Figure 22 shows the pollen tubes in this cross from a 12-hour test of a pervious year at 17°C. It seems that here we have high pollen germination and favorable pollen-tube growth, conditions closely resembling $2n \times 2n$ crosses. It is difficult to explain why this cross should be more favorable than the combination $n \times n$, aside from the non-germinating part of haploid pollen. The data here, however, are based on only a single test and a small population. On the whole, the data seem to indicate that the apparently good pollen grains of Datura which have been derived from a diploid are better than the similar grains derived from a haploid.

HAPLOID × TETRAPLOID CROSSES

Figures 23 and 24 show diagrams of $n \times 4n$ tests. Here we have a high degree of incompatability, for nearly all of the pollen tubes burst within the style, a condition essentially similar to the $2n \times 4n$ crosses. Also, the proportion of the pollen which fails to germinate is around 12–17 percent as it is in $2n \times 4n$ crosses. The sterility of this combination is likewise due to difficulties in pollen-tube growth.

DISTANCE OF PENETRATION OF POLLEN TUBES

It will be noted that the distance reached by the longest pollen tube in a given time is very nearly the same, regardless of the style used and the pollen. The differences shown in some of the tests are due to the differences in time and temperature. When the temperature and time intervals are the same, the distance reached by the longest pollen tubes is nearly the same. Slight differences are always found in tests with styles and pollen of the same origin (BUCHHOLZ and BLAKESLEE1927b) and compared with these variables any differences found in the series of studies would not appear to be significant.

DISCUSSION

Abnormal pollen tubes and conditions influencing pollen-tube growth

There are at least two conditions giving rise to an abnormal swelling and bursting of pollen tubes which must be recognized in these tests of pollen-tube growth between balanced chromosomal types. One is the growth of otherwise normal gametophytes in an unsuitable style. This condition is illustrated by the crosses $2n \times 4n$, $3n \times 4n$ and $n \times 4n$, and appears very similar to the condition found when certain interspecific crosses are attempted in which the primary cause of sterility is definitely

EXPLANATION OF PLATE 1

Photomicrographs of preparations of the conducting tissue containing pollen tubes, dissected from the styles. The preparations were stained in an aqueous mixture of Fuchsin S, 10 parts, and Light Green S. F., 3 parts, cleared in lactic acid, and spread out by pressure on the cover glass.

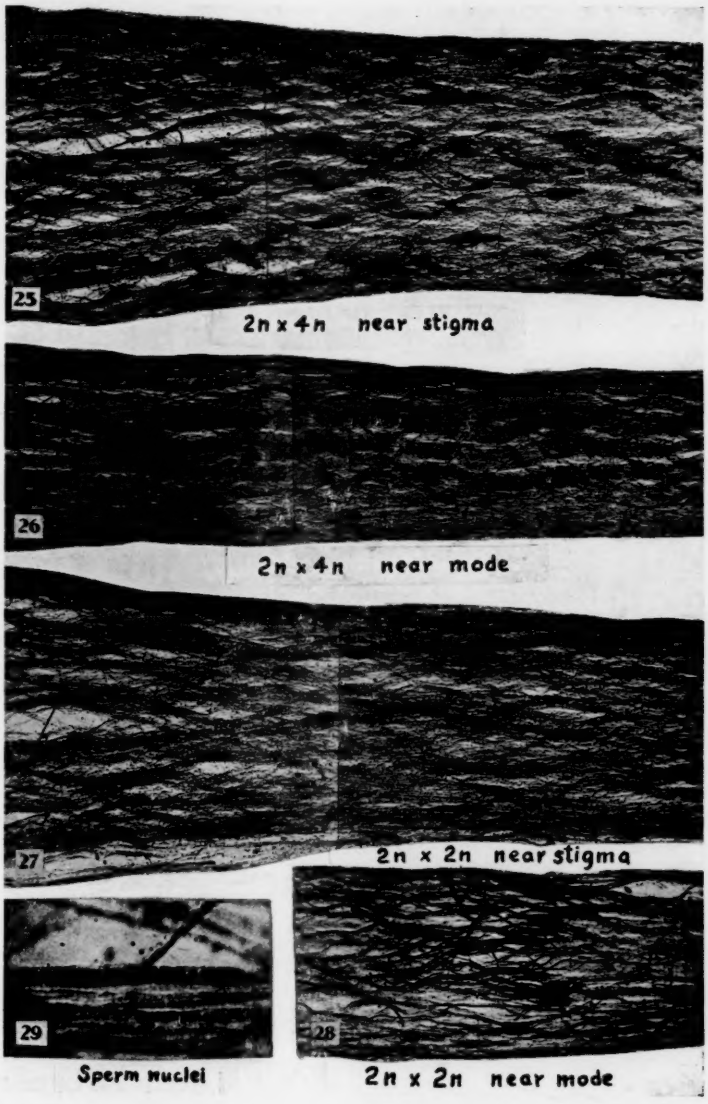
FIGURE 25.—$2n \times 4n$ pollination. Upper portion of style near stigma showing many swollen or burst pollen tubes. ×16. See figure 7.

FIGURE 26.—$2n \times 4n$ pollination. Same style as figure 25 in region of longest pollen tubes. ×16. See figure 7.

FIGURE 27.—$2n \times 2n$ pollination. Upper portion of style near stigma showing only a few swollen or burst pollen tubes. ×16. See figures 1–3.

FIGURE 28.—$2n \times 2n$ pollination. Style similar to figure 27 in the region of mode of distribution of pollen-tube ends. ×16. See figures 1–3.

FIGURE 29.—Two sperm nuclei showing in a pollen tube from a preparation stained in Delafield's Haematoxylin. ×300.


25
2n x 4n near stigma
26
2n x 4n near mode
27
2n x 2n near stigma
29
28
Sperm nuclei
2n x 2n near mode

recognizable as due to difficulties in pollen-tube growth (BUCHHOLZ and BLAKESLEE 1927b). In these crosses, the pollen germinates on the stigma, but the growth of the pollen tubes is followed by swelling and bursting so that sooner or later all or nearly all of the male gametophytes are eliminated. Since the result is much the same whether $1n$, $2n$, or $3n$ styles are used, the cause of the bursting in such cases must be due to something in the styles unsuitable to the growth of pollen tubes having $2n$ chromosomes.

The other condition which causes bursting of pollen tubes seems to depend upon the nuclear constitution of the gametophytes. In self-pollinations or sib crosses between tetraploids, the high non-disjunction which is known to occur in microsporogenesis is reflected in a correspondingly high proportion of burst pollen tubes, and in a high proportion of ungerminated pollen. Similarly, the small proportion of abnormal pollen tubes found in diploid tests ($2n \times 2n$) is probably due in part to differences arising through non-disjunction in the formation of microspores. We know from other pollen-tube tests of the $(2n+1)$ chromosomal mutants that similar abnormal tubes are the result of the extra chromosome, and that this chromosomal abnormality may express itself in various other ways; for some by giving rise to ungerminated pollen grains, and for other chromosomes, to pollen tubes which may remain normal-appearing but grow at a retarded rate. The bursting pollen tubes which result from these two conditions, namely, unsuitable styles, and nuclear constitution of the gametophytes, are not distinguishable from their appearance, and it is from our knowledge of the origin and constitution of the pistil and pollen employed that we are able to distinguish between these two conditions which may cause bursting.

Still other conditions may cause a bursting of a part of the pollen tubes in a population, for instance, the presence of certain genes and this condition has been demonstrated for the gene tricarpel (BUCHHOLZ and BLAKESLEE 1927b.) Presumably our plants are homozygous and we are not concerned with gene differences in these tests unless they should represent lethals which arose anew in considerable numbers.

The most favorable conditions of pollen-tube growth are found in $2n \times 2n$, $4n \times 2n$, $3n \times 2n$ and $n \times 2n$ combinations. In all of these combinations, when pollen of diploids is employed, more than 93 percent of the pollen germinates on the stigma, and the bursting of pollen tubes within the styles occurs only in low proportions. A large number of the pollen tubes are grouped into a pronounced mode near the advancing front, and any outstanding differences in the shape of the polygon of distribution appear to be due to differences in the amount of crowding.

Another series of very favorable conditions of pollen-tube growth is found in the crosses $2n \times n$, $4n \times n$ and $3n \times n$ when pollen of haploid plants is employed. While 23–42 percent of the matured pollen grains from haploids fail to germinate, the portion which does germinate is otherwise similar to the pollen of $2n$ plants, and shows very favorable conditions of pollen-tube growth. The combination $n \times n$ seems to show somewhat unfavorable conditions of pollen-tube growth though some seeds are produced on haploids.

Less favorable conditions of pollen-tube growth are found in tetraploids, in both selfs and sib crosses $4n \times 4n$. The pollen of these is encumbered with many individual abnormalities, but the 45 percent or less of the pollen tubes remaining after the abnormalities are sifted out are fully potent and are able to accomplish fertilization. The $4n$ plants have always been found fully capable of maintaining themselves as distinct tetraploid lines. However, modified tetraploid types are known to occur in cultures, plants of $(4n \pm 1)$, $(4n+1-1)$ and other abnormal constitutions (BLAKESLEE, BELLING and FARNHAM 1923), and if recognized, the use of these should be avoided in making tests of this kind. Some of these may be suspected on account of abnormalities in external appearance and habit, by their failure to set fruit or by having only small seed capsules. They may be positively identified only by cytological methods, and this was done for a few greenhouse plants, which gave results similar to the garden plants used.

Among the garden plants in which special cytological examinations of the plants tested were not made, no abnormal somatic conditions were observable, and none of the pollen-tube preparations obtained from the use of tetraploid plants over a period of several years showed more favorable conditions than the tests obtained in 1927 and used in this study.

The cross $4n \times 2n$, in which pollen-tube growth is favorable, is highly sterile because of difficulties in fertilization, or difficulties in the development of $3n$ zygotes, while in the cross $2n \times 4n$ the difficulties of pollen-tube growth are added to these other barriers to crossing. Thus $4n$ plants are almost completely isolated biologically from $2n$ plants.

In all combinations in which triploid pollen was tested, this pollen was found to be highly impotent, all but a small fraction of it being incapable of germination. The chief difficulties in all pollinations with this pollen are attributable to the abnormal chromosomal conditions, in that most of the pollen grains have several extra chromosomes. The proportion of normal-appearing pollen tubes, presumably with 12 chromosomes is between 1.2 percent and 1.7 percent whether grown on $3n$ or $2n$ styles.

Pollen germination

In all of the combinations in which the pollen of $2n$ plants was employed the germination was well above 93 percent. We take this fact to indicate that the experimental conditions of pollen germination on cut flowers were very favorable; they were much more favorable in fact then similar tests on plants growing in the garden.

When the pollen of $4n$ plants was employed, its germination was only about 75 percent on $4n$ and $3n$ stigmas, and about 85 percent on $2n$ and n stigmas. We are, at present, unable to account for the difference. The additional pollen tubes produced by the higher germination in these crosses probably only increases the proportion of abnormal or burst pollen tubes in the population.

The pollen from triploids is always highly impotent on all stigmas and this is because it gives a very low germination. This pollen was also tested on $4n$ stigmas with no better results than on $3n$ or $2n$ stigmas, and there is no reason to suspect that it might be better on the stigmas of haploids, a test which we have not yet made. The difference between figure 12 and figure 13 may be due at least in part to an experimental error. In the tests of former years represented by figure 12 no special precautions were taken to prevent the loss of ungerminated pollen from the stigma during staining and mounting. Figures 13, 14 and 27–211–1, in fact, all tests made in 1927 were handled in a manner which would prevent or minimize the error from the loss of ungerminated pollen grains remaining on the stigma.

Conditions in haploids

When the pollen from haploid plants was employed, the tests showed from 23–42 percent (general average 32 percent) of the matured pollen ungerminated. This is true without exception of all other tests of this pollen made in previous years. A few of these former tests were included, but most of the preparations had faded so badly that the pollen tubes were too obscure to be counted accurately and these slides were therefore not counted and tabulated. With regard to the relative proportion of ungerminated pollen on these old slides, they show from inspection that there is always a similar large proportion of matured pollen grains which fails to germinate.

The cause of non-germination of a large proportion of the pollen of n plants is not fully understood. The bursting pollen tubes are also present in small proportions in tests employing pollen of n plants and may be attributed to similar causes. These might represent new genes, causing

abnormalities in the pollen, and therefore wholly or partially lethal. The gene tricarpel (BUCHHOLZ and BLAKESLEE 1927a) had its origin in a haploid plant, and though it is transmitted through the pollen, its transmission is accomplished with a high pollen-tube mortality.

Three hypotheses suggest themselves as offering possible explanations. On the hypothesis that these abnormal pollen grains and pollen tubes represent gene mutations, they would be classifiable as lethals, genes which are automatically sifted out in the processes of gametophytic selection. MULLER and ALTENBURG (1919) have shown that lethals occur in Drosophila much more abundantly than genes which survive to become visible. Our Datura records to date for genes derived from a haploid plant (BLAKESLEE, MORRISON and AVERY 1927) have given between 2 and 3 percent genes which survive developmental selection and give visible effects in the sporophyte generation. The proportion of visible genes to lethals or those not transmitted with recognizable regularity would therefore have to be in the proportion of 1:11, 1:12 or more, if this hypothesis accounts for all of the 32 percent non-germinating pollen grains.

It might be urged against this hypothesis that relatively few visible gene mutations are known in Datura from any source (BLAKESLEE, MORRISON and AVERY 1927), and this hypothesis would assume a surprisingly high basic mutation rate. The basic mutation rate, however, has been found to be very much lower in diploids. The various processes of developmental selection (BUCHHOLZ 1922, BUCHHOLZ and BLAKESLEE 1922, 1927a) which are gradually becoming more fully understood and appreciated, offer a possible explanation for their continued elimination of new genes and thus may account for the general scarcity of easily recognized gene mutants in Datura. It is possible that in addition to a higher basic mutation rate in haploid plants we have a less severe elimination of new genes in an n plant under the special conditions at the time when it is producing its $2n$ offspring.

Another possible explanation for these non-germinating pollen grains is that they represent gametophytes with chromosomal deficiences, some of the microspores with less than 12 chromosomes arising from pseudo-reduction.

If even a small proportion of the total product of pseudo-reduction posessed only 11 chromosomes and yet was capable of reaching maturity, this class might be of considerable significance as a component of the matured grains in view of the high amount of pollen abortion. However, we have assumed that chromosomal deficiences result in abortive pollen,

since all $(2n-1)$ deficiences (chiefly sectorial branches) thus far recognized have produced at least 50 percent abortive pollen (BLAKESLEE and CARTLEDGE 1926, 1927). Thus, in an anther of a flower from a $(2n-1)$ branch, in which the output of pollen with 12 chromosomes is 50 percent, gametophytic selection in the pre-pollination stage apparently eliminates half of the pollen output within the anther or all of the pollen grains with 11 chromosomes. A third possible explanation is that these ungerminated pollen grains, together with the pollen tubes which burst in the style, represent pollen grains with 13 or 11 chromosomes, brought about by non-disjunction in sporogenous tissue. BELLING and BLAKESLEE 1926, report two such cases. Non-disjunction in the division following non-reduction would also give rise to pollen grains with 13 and 11 chromosomes. That such non-disjunctions may occur in haploids is suggested by the relatively high percentages of non-disjunctional types produced by a haploid (BLAKESLEE, MORRISON and AVERY 1927), although their occurrence might be due in part to non-disjunction in the early zygote, which therefore could not be of significance in connection with the pollen. Any non-disjunction associated with non-reduction might be of considerable significance in haploids due to the large proportion of grains which abort, as has been suggested in the preceding paragraph.

On the basis of known facts, it is possible that all three hypotheses are to some degree correct. There is good reason to believe that both the abnormal pollen tubes and the non-germinating pollen grains produced by a haploid plant are in some way connected with a high number of spontaneous chromosomal and gene mutations found in haploid progeny.

Developmental selection

The process of developmental selection, which has been alluded to in previous paragraphs, has been discussed in previous publications (BUCHHOLZ 1922, BUCHHOLZ and BLAKESLEE 1922, 1927a). These experiments on Datura serve as further illustrations of some of the specific eliminating effects of this mechanism of evolution.

We are concerned with many of the forms of developmental selection in these experiments. In angiosperms, the most important of these are interovular and gametophytic selection. Interovular selection brings about eliminations between ovules within the same ovary; (a) after the fertilization stage. This form of interovular selection is illustrated by the high mortality among the enlarged ovules or developing seeds of Datura resulting from the cross $4n \times 2n$, or the high selective mortality from the

cross $3n \times 2n$. In the cross $4n \times 2n$, there are obtained only a few viable seeds per capsule plus very many abortive ovules which had begun to enlarge but which failed to mature. The term interovular selection implies that the ovules containing individuals lost during seed development may have been crowded out during embryonic stages in competition with other ovules containing more vigorously growing individuals. Of course, many zygotes are lethal due to their genetic constitution and might not be able to survive under the most favorable conditions. Enlarged ovules which are eliminated may include those in which fertilization was not complete, resulting in an embryo without endosperm or endosperm without a fertilized egg, as well as those containing perfect embryos. An ovule containing an embryo derived by parthenogenesis would tend to be eliminated if this weaker individual is growing in competition with $2n$ zygotes in the ovary of a $2n$ plant. On the other hand, the parthenogenetic individuals which are produced in the seed capsule from a $4n \times 2n$ cross, where they would be $2n$ (or $2n \pm 1$), would probably be among the survivors, as our genetic data indicate.

Interovular selection may also take place: (b) before the fertilization stage, bringing about a selective elimination between ovules within the same ovary, probably due to the type of female gametophytes which they contain. We have observed many ovules which had pollen tubes extending into the micropyles but which had remained unenlarged at a stage when neighboring ovules had increased their volume many times and contained embryos. The detailed study of these early aborting ovule types presents some difficulties, and we have not pursued our investigations of these small aborted ovules beyond the point of definitely ascertaining the fact that these stages of elimination exist.

Gametophytic selection is of two forms or phases: (a) between female gametophytes; and (b) between male gametophytes. That which may occur between female gametophytes in Datura within the same ovule (that is, between the four megaspores or their products) has not been investigated. Our experimental studies have been confined very largely to a study of (b) the gametophytic selection in male gametophytes. We have come to distinguish between two stages of the latter: gametophytic selection between male gametophytes within the anthers before the maturity of the pollen; and gametophytic selection between male gametophytes after the pollen reaches the stigma. This latter stage is most successfully subjected to experimental study and our investigations have been directed chiefly toward a better knowledge of this form of developmental selection.

The gametophytic selection which takes place within the anthers during the development of the pollen grains from microspores is a form or phase of developmental selection. As pointed out in the previous pages, a 24-fold enlargement, accompanied by a nuclear division, takes place during these stages, and in some types many microspores are eliminated as aborted pollen. The great range in the sizes of the aborted pollen grains of triploids and haploids indicates that eliminations occur in all stages from the smallest microspores on to the stages approaching pollen maturity. The facts seem to indicate that, on the whole, this pre-pollination stage of gametophytic selection eliminates most largely the deficiency gametophytes with less than 12 chromosomes, while the gametophytes with extra chromosomes are most largely eliminated by gametophytic selection after pollination.

The phase of gametophytic selection which takes place among male gametophytes after pollination is one of the most important stages in which eliminations occur. This is well illustrated by the tests involving the use of triploid pollen in which the small proportion of pollen tubes with 12 chromosomes are effectively sifted from a large mass of pollen grains with excessive chromosomes. Only a few types of $(2n+1)$ plants are known to exist in which an extra chromosome may be transmitted through the pollen, and in these instances, the pollen tubes grow at a slower rate. It is in pollinations using the pollen of these $(2n+1)$ types that we have had some success in our efforts to control the selection of male gametophytes, through manipulations which tend to crowd or not crowd the pollen tubes, by cutting off previously pollinated styles at the proper time, and other similar methods.

Likewise in tetraploids $(4n \times 4n)$ the non-germination of 25 percent of the pollen and the early elimination of another 30 percent of the pollen tubes are important means which limit the transmission of newly acquired chromosomal abnormalities, while there is abundant evidence that interovular selection also eliminates a high proportion of abnormal female gametophytes and zygotes.

The effect of the various processes of developmental selection in tetraploids and diploids is, therefore, to render them more or less stable. Tetraploids have been maintained for many years in cultivation as distinct races from generation to generation. Triploid and haploid types are not capable of maintaining themselves; the same processes operate to render these types unstable. In these types, developmental selection tends to eliminate or hinder the reproduction of their kind. Triploids produce relatively few seeds, and may give rise to diploids,

tetraploids, and various $(2n+1)$, $(2n+1+1)$, types rather than $3n$ plants, while haploids give rise to diploids, and to $(2n+1)$ chromosomal types (BLAKESLEE, MORRISON and AVERY 1927), rather than to haploid types. In addition a haploid induces a relatively high proportion of visible gene mutants. There seems to be good evidence that a much higher proportion of gene and chromosomal abnormalities (perhaps 10-12 times as many) are produced in the pollen of a haploid than can become recognizable in the adult plant.

SUMMARY

1. Pollen-tube growth was studied by means of tests made under controlled conditions, using a technique described elsewhere. Pollen-tube distribution curves and statistical tables were prepared showing the condition and distribution of the ends of pollen tubes in the style after comparable time intervals.

2. Abnormalities in pollen-tube growth were found in all plants investigated. These consisted of swollen ends and burst pollen tubes within the style. These occurred only in smaller numbers in selfs and crosses among $2n$ plants and in the styles of any type pollinated with pollen having 12 chromosomes. When these occurred in excessive proportions, this is regarded as an indication of unfavorable conditions of pollen-tube growth.

3. A high proportion of ungerminated pollen remaining on the stigma is an indication of unsuitable genetic constitution of the pollen.

4. The nature of the style and stigma is an important contributory cause of the abnormalities in pollen-tube growth of certain combinations. Styles of n, $2n$, and $3n$ plants were found unsuited to the growth of pollen tubes coming from the pollen of $4n$ plants. Styles of all types, n, $2n$, $3n$, and $4n$ were found to be favorable for the growth of pollen from $2n$ plants.

5. The pollen of tetraploid plants gave only about 75 percent germination on tetraploid stigmas, and about 85 percent germination on $3n$, $2n$ and n stigmas, where the pollen-tube growth within the style is unfavorable.

6. Abnormal pollen tubes due mainly to bursting were found in considerable numbers in $4n \times 4n$ tests, in which pollen-tube growth was most favorable. This corresponds quite closely to the high non-disjunction in microsporogenesis of tetraploids, which results in many pollen grains with 23 and 25 chromosomes.

7. The pollen of triploid types gives very little germination, either

on their own type of stigma or on that of $2n$ and $4n$ types. Only about 10 percent of this pollen is capable of germination, and about 1.5 percent of the pollen grains produce tubes which may be presumed to have 12 chromosomes. This percentage is about 30 times that expected on the basis of random assortment found at reduction and suggests unknown factors affecting chromosome numbers.

8. From 60-70 percent of the small proportion of the pollen grains which mature in a haploid may germinate on the stigma of $4n$, $3n$, $2n$ and n types. The pollen tubes within the style have a smaller proportion of burst tubes and present growth conditions otherwise similar to $2n \times 2n$ tests, except for the tests $n \times n$.

9. Three hypotheses are proposed in explanation of the nature of the ungerminated portion of the pollen from haploids. Of these, the suggestion that some may contain new genes which are lethal in the male gametophytic stage, and the suggestion that some may be pollen grains with 13 chromosomes, is in line with genetical evidence; another suggestion that some may be pollen grains with 11 chromosomes arising from pseudo-reduction is improbable but not entirely disproved.

10. The nature and role of developmental selection in relation to the types of offspring produced by the n, $2n$, $3n$, and $4n$ types is discussed. While the actual origin of the chromosomal types depends upon cytological processes, developmental selection in its different forms serves as a sieve which determines the type of offspring which may survive to the next generation and modifies the proportions in which these types may appear.

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AN ENZYME DIFFERENCE ASSOCIATED WITH THE WAXY GENE IN MAIZE.[1]

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INTRODUCTION

The waxy character in maize is inherited as a simple recessive to the non-waxy condition. It manifests itself in the pollen and in the endosperm. If the pollen of waxy plants is treated with a little dilute iodine the granular reserve materials assume a yellowish or reddish-brown color. Pollen from non-waxy races of maize gives a bluish reaction with the same reagent. The difference observed is due to the kind of starch present. The reserve starch found in the non-waxy race is the common sort while that in waxy plants is in some way different, as the iodine test reveals. This peculiar carbohydrate is likewise formed in the endosperm of the waxy race and may be prepared in the laboratory in quantity from the latter tissue. In size and form the starch grains from waxy and non-waxy seeds are indistinguishable. It is not until they are stained with iodine that a difference becomes evident.

An interesting situation is presented in the pollen of the hybrid between a waxy and a non-waxy plant. The crossbred plant produces pollen grains 50 percent of which stain bluish and 50 percent, brownish. In their color reaction to iodine, therefore, one-half the pollen grains are like those of the waxy parent and the other half are identical with those of non-waxy plants. The one-to-one ratio of waxy and non-waxy pollen grains is interpreted as an immediate result of the segregation of the corresponding genes. We thus have a clearly discernible effect of these genetic factors in the gametophyte, one cell generation removed from the

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reduction divisions. The prompt differential action of the waxy factor in the pollen suggested to us that favorable material might be offered here for a study of the gene as a physiological unit.

It may be assumed that the waxy and non-waxy allelomorphs impinge upon a reaction or series of reactions leading in the one case to the production of the brownish-staining starch and in the other to common starch. Our task is to trace out the chain of physiological processes linking character and gene with a view of determining the mechanism by which the gene operates.

The first step in such an analysis is a study of the difference in the end products formed under the action of the allelomorphic genes. The reserve carbohydrate formed in waxy seeds is insoluble in water and in 10 percent alcohol. It is to be looked upon, therefore, as a starch and not as a dextrin (BRINK and ABEGG 1926). How does it differ from common maize starch? Starch is a complex substance whose chemical structure is little understood. Some advances, however, have been made in recent years. The elementary starch molecule consists wholly of glucose residues. According to the present view the number of glucose residues is small but there is no agreement as to the exact number involved or the manner in which they are linked together. It is assumed that the elementary starch molecules are either polymerized into a higher unit or bound together by some force other than chemical valency.

In view of the somewhat greater solubility of waxy starch it appears likely that this substance is less highly polymerized or "associated" than common maize starch.

An attempt has been made to carry the analysis further to determine if the elementary molecules of which the two starches are composed are alike. As the exploring agent a standard preparation of malt diastase has been employed. It was desired to determine two things, (1) the ultimate products of diastatic hydrolysis, and (2) the rate at which the conversion takes place.

To be brief, it was found that on hydrolysis both waxy and common maize starch gave an almost theoretical yield of the same sugar—maltose. A trace of glucose was present in both cases. These sugars were identified in the usual ways, namely, by their reducing powers, their specific rotations and the crystalline forms of their phenylhydrazine derivatives. No *iso*maltose, which some investigators contend is formed on treating starch with diastase, could be detected. The fact that both starches

yield only maltose indicates that the fundamental building stones of which they are composed are identical.

It was found, however, that the rate at which maltose is formed from the waxy and non-waxy starches under the action of malt diastase is different. Under identical conditions maltose is produced somewhat more rapidly from the common maize starch than from the waxy starch. This may mean that, while the sugar units composing the starch molecules are alike, the mode of union of these sugar residues is different in the two cases. Possibly there is a difference in stereoisomeric configuration, the linkings in the case of waxy starch being less easily opened by malt diastase than those of common starch (BRINK 1928).

In seeking an agency which might have caused this apparent difference in structure of waxy maize starch one's attention naturally turns to the enzyme, amylase, which is concerned in starch synthesis and hydrolysis. It has been suggested frequently that enzymes may be the intermediate agencies through which genes produce their ultimate effects. ONSLOW (1915) reports that chloroform water extracts of the skins of recessive white and yellow rabbits lack tyrosinase whereas similar preparations from black, chocolate and blue skins contain it. Enzymes have been postulated to account for gene action in numerous other cases but direct experimental verification has either not been attempted or tests have failed to confirm the hypothesis (WHELDALE 1916; KEEBLE and ARMSTRONG 1912).

In the present series of experiments the diastatic activity of comparable lots of waxy and non-waxy seedlings, seeds and pollen has been measured. Special care has been taken to arrange the conditions of the tests in as simple fashion as possible. The dried seedlings, seeds and pollen have been ground thoroughly and the enzyme extracted from the powdered material with water. No attempts to purify the amylase were made. Much handling would be necessary to do this and there is always the possibility that the reagents used will modify the activity of the enzyme. The crude extracts were tested directly on substrates of three kinds, potato, waxy and non-waxy maize starch pastes.

THE AMYLASE OF WAXY AND NON-WAXY SEEDLINGS

The two classes of seed on a series of well-matured, healthy ears segregating for waxy were separated by inspection and germinated on moist paper at room temperature. When the radicles had attained a length of about one-quarter inch the seedlings were removed from the moist pad and dried in an oven at 40°C. to constant weight. The dried

seedlings were then ground in a pestle mill until the particles passed a 40-mesh sieve. The powdered material was thoroughly mixed and stored in stoppered bottles at room temperature. In test 1, 5 grams of each lot of the powdered seedlings were suspended in 25 cc of water. The mixtures were stirred frequently during the first few hours and, after a period of 24 hours and 25 minutes, were filtered through a single layer of cheesecloth to remove the larger particles. In measuring the hydrolytic activity of these extracts 15 cc were measured out and added to the starch paste. The enzyme extracts used in test 2 were prepared in substantially the same way except that 20 grams of the powdered material were treated with 50 cc of water for 15 hours and 15 minutes and 30 cc of each of two respective filtrates employed in the conversions.

In preparing the substrate 25 grams of air dry potato starch were gelatinized by heating with water and made up to a volume of 600 cc after cooling. The acidity was adjusted with acetic acid to approximately pH 4.5. The starch paste was then divided, 250 cc being measured into each of two flasks to which the respective fresh enzyme extracts were added. The conversions were carried out on a water bath at 33° C. A few drops of toluol were added to each flask as an antiseptic.

Preliminary examination showed that the sugar formed on treating starch with a maize seedling extract prepared in this way is glucose. Since alcohol was not used it was to be expected that the crude water extract would contain active maltase as well as amylase. The osazone test applied revealed clearly that hydrolysis was carried to the glucose stage. Accordingly the reducing power is computed in terms of this sugar.

Samples of the conversion liquid were taken at the intervals shown in tables 1 and 2 and inactivated by boiling. The amount of glucose present was measured by the iodimetric method of BAKER and HULTON (1920).

The results of tests 1 and 2 are shown in tables 1 and 2. The values obtained in test 1 have been plotted in figure 1 using the arith-log scale in order that a direct comparison of the rates of hydrolysis may be made. It will be recalled that on this ruling the same *percentage* difference is always represented by the same vertical distance. Equal rates of change are indicated by lines of equal inclination. Tests 1 and 2, which were alike except that the concentration of enzyme was higher in the latter, are entirely in agreement in showing that the amylase extracts of waxy and non-waxy seedlings hydrolyse starch at the same rates. During the first four hours digestion is rapid. The speed of the reaction then falls off

quickly and after about 14 hours diminishes to a low value which remains nearly constant up to 40 hours, at which time the experiments were terminated. Throughout their entire course the waxy and non-waxy curves practically coincide.

TABLE 1

Amounts of glucose formed on treating potato starch paste with amylase extracts from ground non-waxy and waxy seedlings respectively. Test 1.

TIME	PERCENTAGE GLUCOSE IN SOLUTION	
	NON-WAXY EXTRACT	WAXY EXTRACT
15 minutes	0.53	0.59
48 "	0.86	0.90
3.5 hours	1.57	1.58
6 "	1.72	1.74
10 "	1.89	1.90
14 "	2.00	2.00
19 "	2.07	2.07
27 "	2.13	2.08
40 "	2.12	2.18

TABLE 2

Amounts of glucose formed on treating potato starch paste with amylase extracts from ground non-waxy and waxy seedlings respectively. Test 2.

TIME	PERCENTAGE GLUCOSE IN SOLUTION	
	NON-WAXY EXTRACT	WAXY EXTRACT
15 minutes	0.55	0.59
45 "	0.83	0.87
3 hours	1.51	1.68
8.5 "	1.73	1.76
11 "	1.84	1.87
15 "	1.89	1.96
21 "	2.01	2.10
27 "	2.06	2.13
40 "	2.32	2.33

THE AMYLASE OF WAXY AND NON-WAXY SEEDS

When the dry mature seed of maize is ground and treated with water an extract is obtained possessing a low but measurable diastatic activity. It has been shown that the endosperm of the corn grain, separated from the embryo, undergoes autodigestion if suitable conditions of moisture and temperature are maintained. This hydrolysis is to be attributed,

presumably, to such amylase as is retained in an active condition after the protoplasm of the endosperm cells ceases to function. It apparently represents the residue of the amylase concerned in the synthesis of the endosperm starch reserves. During germination its influence is probably slight as compared with the much larger amounts of amylase secreted by the awakening embryo.

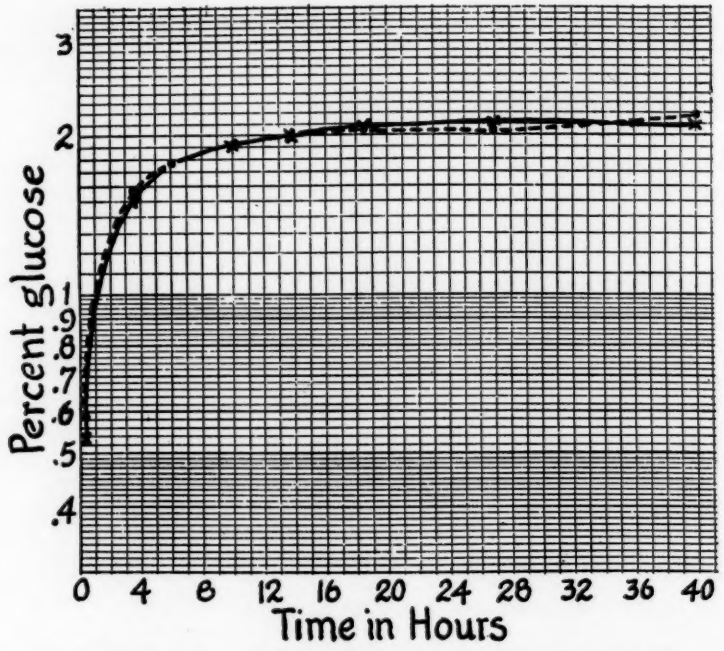

FIGURE 1.—Rate of glucose formation from potato starch paste under the action of amylase extracts of waxy and non-waxy seedlings in test 1. Broken line, waxy; solid line, non-waxy.

If it could be assumed that during the ripening of the seed and the subsequent period of storage no differential inactivation of this residual amylase occurs, a study of the diastatic activity of waxy and non-waxy seeds should throw light on the influence of the waxy gene on the character of the amylase concerned in starch synthesis.

Ten tests on the diastatic power of water extracts of the seed have been carried out. The grain was taken from segregating ears as before

and ground finely without separating embryo and endosperm. The material was stored in corked bottles at room temperature and tested during the two weeks subsequent to grinding. Weighed amounts of meal were treated with water for periods varying from 9 to 22 hours and the extract, after filtering through cheesecloth, tested on substrates of corn starch paste in some trials and potato starch paste in others.

The experiments revealed, without exception, on both kinds of substrates that the extract from the waxy seeds possessed a somewhat higher diastatic activity than those from the non-waxy seeds. The results of test 3, shown in table 3, are presented as typical of the series. The substrate

TABLE 3

Amounts of glucose formed on treating waxy starch paste with water extracts of ground waxy and non-waxy seeds. Test 3.

TIME IN MINUTES	PERCENTAGE GLUCOSE IN SOLUTION	
	NON-WAXY EXTRACT	WAXY EXTRACT
20	0.37	0.43
50	0.42	0.49
120	0.48	0.57
360	0.62	0.75
540	0.74	0.94

used was waxy maize starch in a concentration of approximately 4 percent. The acidity was adjusted with acetic acid to pH 4.5. The enzyme extracts were prepared by suspending 20 grams of the powdered whole grain in 50 cc. of water for 22 hours. Thirty cc. of the filtered extract were added to 250 cc. of starch paste and the conversion carried out at 50°C. It will be noted from figure 2 where the result of test 3 is shown in graphical form, that at 20 minutes the amount of glucose formed in the sample treated with waxy amylase is clearly higher than that in the other lot. The difference in rate from this point on is small but slightly in favor of the waxy sample.

During the course of these experiments it was observed that, whereas the extract of the freshly ground seeds showed a difference in diastatic power in favor of the waxy lot, the disparity was slightly but significantly increased as the meals aged. Evidently a secondary change was occurring affecting the factor being measured. The question arose at once whether the alteration might not be a continuation of some process going on in the intact seeds as well. If such were the case, the initial difference in diastatic activity of the seeds might have been so altered during the period

of ripening and storage that the present measurements would be valueless for our purpose.

About the time these observations were made Dr. F. A. ABEGG, working in our laboratory, discovered that waxy and non-waxy seeds dried and stored in the ordinary way differed considerably in free fatty acid content. The acid value[2] of the ether extract of freshly ground non-waxy was found to be 5.7; that from the waxy seed, on the other hand, was 23.4. The fat extracted from the two classes of embryos was found to be practically neutral. The difference lay in the comparatively

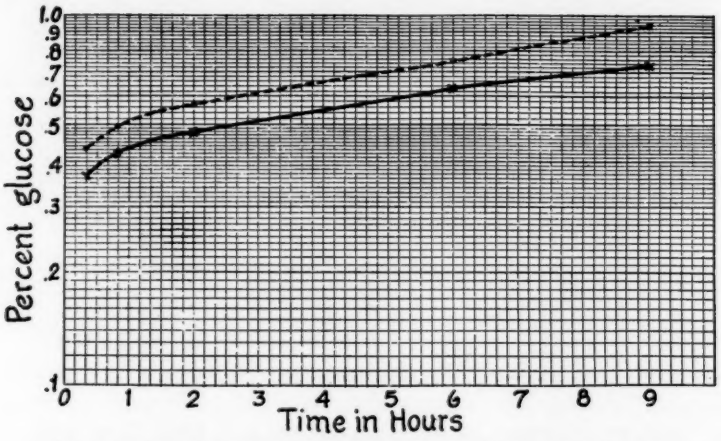

FIGURE 2.—Rate of glucose formation from potato starch paste under the action of amylase extracts from waxy and non-waxy seeds in test 3. Broken line, waxy; solid line, non-waxy.

small amount of fatty substance in the endosperms. The ether soluble extract from non-waxy endosperm tissue showed an acid value of 45 whereas the corresponding figure for the waxy endosperm was 82.

ABEGG observed, further, that during storage in bottles at room temperature, the acid values of the whole grain meals increased greatly. During a period of four months a sample of non-waxy meal rose in acid value from 10 to 39 and a corresponding lot of waxy meal from 30 to 106. These are increases of over 250 percent.

It will be recalled that the difference in the character of the carbohydrate reserves in the endosperms of waxy and non-waxy seeds is likewise ex-

[2] The acid value is the number of milligrams of KOH required to neutralize one gram of the fatty substance.

hibited by the starch in the pollen of the two kinds of plants. An important fact which ABEGG's studies reveal is that the difference in acid values shown by the waxy and non-waxy endosperms does not obtain in the pollen. The ether extracts of waxy and non-waxy pollen both give acid values of about 42. This led ABEGG to conclude that the difference in the proportion of free fatty acids in the endosperm tissues is a secondary effect of the waxy and non-waxy allelomorphs.

Directly or indirectly, the difference in acidity of the waxy and non-waxy endosperms might well affect the activity of the amylase contained therein. The period during which the endosperm amylase is exposed to such modification, moreover, is relatively long compared to that in the pollen. It is doubtful, therefore, whether any considerable dependence is to be placed in the tests on the comparative diastatic activity of endosperm extracts as revealing a primary effect of the waxy gene.

In arriving at our conclusions regarding the mode of action of the waxy gene little weight has been given to the data obtained with seed extracts. It is believed much more significance is to be attached to the facts on diastatic activity of the pollen. Lest our critics should think, however, that facts are being withheld which may appear to run counter to the hypothesis later advanced, the general results on the seed amylases are presented.

THE AMYLASE OF WAXY AND NON-WAXY POLLEN

It is in the pollen that one should expect to obtain the clearest picture of the influence of the waxy gene on amylase production. There is far less opportunity here than in the seed for secondary reactions to obscure the primary effects both during development and in storage. The pollen grain is a simple, undifferentiated structure which early becomes cut off by a heavy wall from the sporophyte producing it. Starch deposition appears to be completed only a few days prior to anthesis so that the enzymes concerned in the process are probably little altered in pollen collected at this time. In contrast with the viable seed, used in previous experiments, the pollen tested has been stored in the non-living condition. Maize pollen is quickly killed by drying and may be preserved over calcium chloride in a thoroughly desiccated condition for a long period of time without losing its diastatic activity.

Comparative tests have been made of the diastatic power of four classes of pollen, non-sugary non-waxy ($S_u W_z$), non-sugary waxy ($S_u w_z$), sugary non-waxy ($s_u W_z$) and sugary waxy ($s_u w_z$). Seed from a number of plants of each class descended by self-pollination from a single $S_u s_u W_z w_z$

progenitor, two generations removed, served as the source of material. This means was taken to balance the effect of genes other than those whose differential action it was desired to measure.

Much difficulty was experienced at first in grinding the pollen thoroughly so that a uniform extraction of the enzyme could be made. The grains are only about 40μ in diameter and the walls are extremely tough. It was found eventually that grinding for 36 hours in a ball mill reduced the pollen to a powder so fine that a single intact grain could not be found. The results reported below are based on tests with pollen thus ground in a ball mill.

The essential conditions under which the several tests on the comparative diastatic activity of waxy and non-waxy pollen were carried out are summarized in table 4. Weighed amounts of the pulverized

TABLE 4

Summary of conditions under which the tests with pollen extracts were made.

KINDS OF POLLEN TESTED	TEST NO.	AMOUNT OF POLLEN EXTRACTED	AMOUNT OF EXTRACT ADDED TO 250 CC STARCH PASTE	SUBSTRATE
S_uW_x, S_uw_x	13	5 gms in 30 cc H_2O	All	Potato starch
" "	15	10 " " 60 " "	"	Waxy corn "
s_uW_x, s_uw_x	16	10 " " 60 " "	"	" " "
" "	17	10 " " 60 " "	"	" " "
S_uW_x, S_uw_x	18	20 " " 50 " "	"	Potato "
" "	19	20 " " 50 " "	"	Non-waxy corn starch
s_uW_x, s_uw_x	20	15 " " 50 " "	"	Non-waxy corn starch
" "	21	15 " " 50 " "	"	Potato starch

The ground pollen was extracted over night for periods varying from 15 to 18 hours in different experiments, except in tests 18 and 19 where the time was extended to 28 hours.

material were mixed with the stated volumes of water and allowed to stand for not less that 15 hours with occasional stirring at first. A few drops of toluol were used to check bacterial growth. Powdered maize pollen absorbs a considerable amount of water and retains it with great tenacity. This occasioned so much difficulty in separating the liquid containing the extracted amylase from the mass that the entire mixture of pollen and water was added to the starch paste.

Pastes prepared from potato starch and waxy and non-waxy maize starches in approximately three percent concentration were used as substrates. The acidity was adjusted with acetic acid to pH 4.5. The conversions were carried out on a water bath at 40°C. As in the previous

tests the reducing power of the samples taken for analysis was measured by the iodimetric method of BAKER and HULTON. On account of the comparatively low diastatic power of the pollen extracts hydrolysis was followed up to 96 or 125 hours.

The osazone test showed that glucose was the only sugar present in the conversion liquids in detectable amounts. The results are consequently expressed in terms of this substance.

Results of tests with S_uW_x and S_uw_x pollen extracts

In table 5 and figure 3 the results are shown of an experiment, designated test 13, on the diastatic activity of S_uW_x and S_uw_x pollen extracts with potato starch paste as the substrate. The rate of hydrolysis in both

TABLE 5

Percentage of glucose formed from potato starch paste through the action of S_uW_x and S_uw_x pollen extracts. Test 13.

TIME	PERCENTAGE GLUCOSE IN SOLUTION	
	NON-WAXY EXTRACT	WAXY EXTRACT
10 minutes	0.010	0.064
1 hour	0.066	0.069
5.5 hours	0.081	0.082
23 "	0.107	0.088
101 "	0.172	0.123

samples is high during the first hour. It falls off very rapidly during the next four and one-half hours in the case of the sample treated with the waxy extract; from this period until the end of the test, which was terminated at 101 hours, the rate is almost constant. The speed of hydrolysis in the non-waxy sample decreases less slowly. Up to five and one-half hours it is doubtful if a real difference between the two lots exists. Samples of the conversion liquids taken up to this time are rather turbid and it is difficult to read the end points in titration with sufficient accuracy to establish certainly such small differences as are shown. From four and one-half hours up to 23 hours, however, the S_uW_x extract clearly shows a greater activity. From 23 hours onward the rate of change appears to become constant at a value slightly higher than that in the S_uw_x lot.

Similar results were obtained in test 18, the results of which are given in table 6. The first pair of samples was taken at 30 minutes and showed almost the same content of glucose. From this point onward the two lots

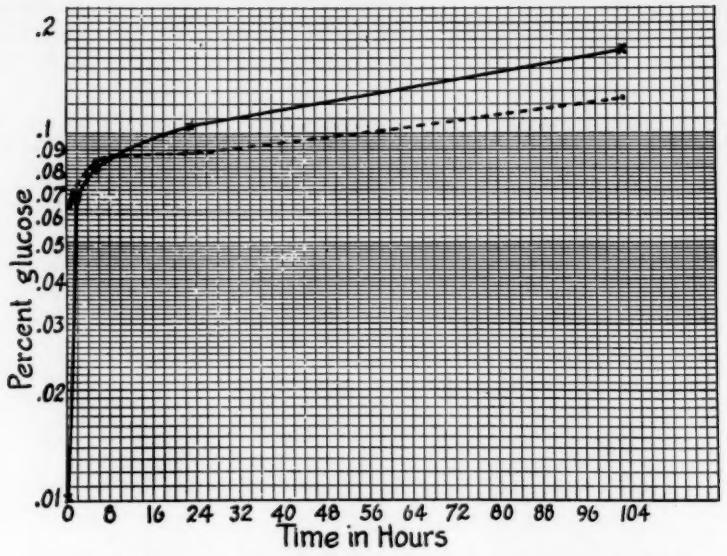

FIGURE 3.—Rate of glucose formation from potato starch paste under the action of amylase extracts of $S_u W_z$ and $s_u w_z$ pollen in test 13. Broken line, waxy; solid line, non-waxy.

TABLE 6

Amounts of glucose formed on treating potato starch paste with $S_u W_z$ and $S_u w_z$ pollen extracts. Test 18.

TIME IN HOURS	PERCENT GLUCOSE ON STARCH	
	NON-WAXY EXTRACT	WAXY EXTRACT
0.5	30.12	30.70
5	39.63	36.89
14.5	44.67	40.35
24	50.29	41.97
48	55.34	45.57
72	60.67	43.81
120	65.43	44.96

draw apart. In both, the rate steadily decreases but less rapidly where the $S_u W_z$ extract was used. At 120 hours over 20 percent more glucose, reckoned on the initial concentration of starch, has been formed under the action of the non-waxy enzyme.

In table 7 and figure 4 the results are shown of a parallel experiment in which waxy maize starch paste formed the substrate. At one hour the amount of glucose formed by the waxy extract is slightly higher but

TABLE 7

Amounts of glucose formed from waxy maize starch paste under the action of $S_u W_x$ and $S_u w_x$ pollen extracts. Test 15.

TIME IN HOURS	PERCENT GLUCOSE ON STARCH	
	NON-WAXY EXTRACT	WAXY EXTRACT
1	16.57	17.72
9	20.03	19.31
25	24.50	21.32
50	28.82	22.19
75	31.85	23.35
100	35.45	24.64
125	39.20	25.79

again the question must be raised as to whether this difference is valid on account of the limitations of accuracy in titrating the turbid solution obtained at first. From eight hours onward to the end of the test at

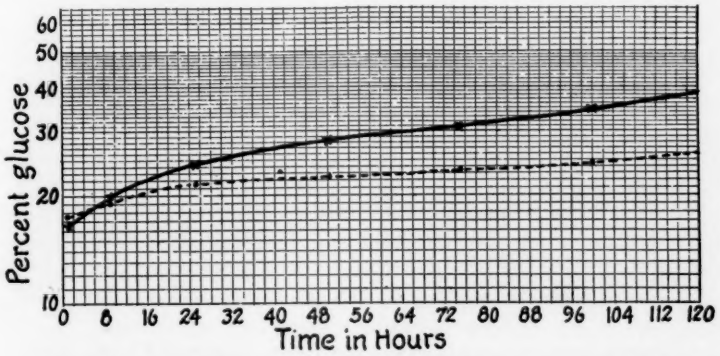

FIGURE 4.—Rate of glucose formation from waxy maize starch paste under the action of amylase extracts from $S_u W_x$ and $S_u w_x$ pollen in test 15. Broken line, waxy; solid, non-waxy.

125 hours the rate in the case of the non-waxy enzyme is consistently higher.

A similar test with the extracts of $S_u W_x$ and $S_u w_x$ pollen on common maize starch yielded the results given in table 8 and represented graph-

TABLE 8

Amounts of glucose formed from non-waxy maize starch paste under the action of S_uW_x and S_uw_x pollen extracts. Test 19.

TIME IN HOURS	PERCENT GLUCOSE ON STARCH	
	NON-WAXY EXTRACT	WAXY EXTRACT
0.33	31.56	35.02
5	41.21	36.74
14	45.40	42.94
24	47.27	44.10
47.5	54.62	45.11
72	57.93	44.82
120	63.70	46.83

ically in figure 5. There are some irregularities in the values obtained from the early samples but the general results are the same as those of the preceding experiments. The water extract from the S_uW_x pollen hydrolyses this starch to sugar more rapidly than does the S_uw_x preparation.

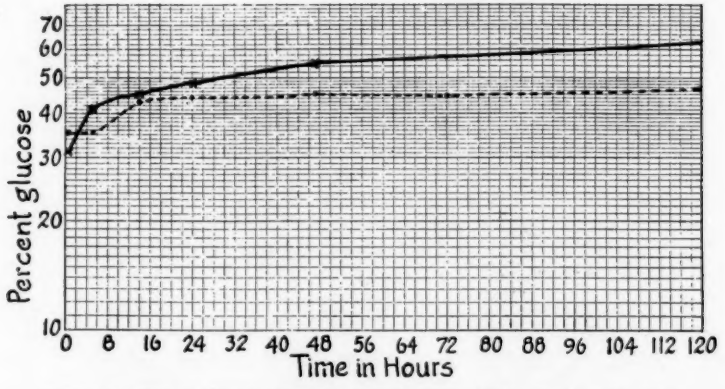

FIGURE 5.—Rate of glucose formation from non-waxy maize starch paste under the action of amylase extracts from $S_u W_x$ and $S_u w_x$ pollen in test 19. Broken line, waxy; solid line, non-waxy.

Results of tests with s_uW_x and s_uw_x pollen extracts

Maize plants heterozygous for the waxy gene (W_xw_x) when self-pollinated or used as pollen parents with the recessive type usually produce fewer waxy seeds than are expected on Mendelian grounds. We have attributed the deficiency to a lower rate of growth of the waxy

pollen tubes, probably in the early stages of development. If the waxy heterozygotes are homozygous for the sugary gene ($s_u s_u W_x w_x$) the disparity in the non-waxy: waxy ratio is significantly increased (BRINK and BURNHAM 1927). In the stocks from which the pollen was gathered for the present enzyme tests the difference in the waxy ratio between self-pollinated non-sugary and sugary waxy heterozygote was about five percent in 1926. The experiments reported below were carried out to determine whether the "exaggerating" effect of the sugary gene on the waxy ratio is reflected in the diastatic activity of the two sorts of pollen.

In table 9 the results are given of a test of the diastatic activity of ground $s_u W_x$ and $s_u w_x$ pollen on a waxy starch paste substrate. The first samples taken at one hour indicate a slightly higher glucose content in the waxy lot but the difference is very small. At 21, 45, 68, and 98 hours, respectively, the amounts of sugar formed under the action

TABLE 9

Amounts of glucose formed from waxy starch paste undert he action of $s_u W_x$ and $s_u w_x$ pollen extracts Test 16.

TIME IN HOURS	PERCENT GLUCOSE IN SOLUTION	
	NON-WAXY EXTRACT	WAXY EXTRACT
1	0.187	0.196
21	0.242	0.221
45	0.251	0.225
68	0.261	0.226
98	0.269	0.241

of the non-waxy pollen extract are greater than for the waxy. After 21 hours the rates of hydrolysis in both cases are almost constant and low. The reaction appears, however, to proceed somewhat more rapidly in the non-waxy than in the waxy lot.

In test 17 a parallel experiment using the same substrate the results of which are presented in table 10 the rate of hydrolysis in the sample treated with the extract of the $s_u W_x$ pollen is higher at all stages. At 96 hours about 10 percent more glucose, reckoned on the initial concentration of starch, has been formed in the non-waxy than in the waxy sample. A very similar result is obtained when potato starch paste rather than waxy starch paste is used as the substrate, as shown in table 11. From the beginning the $s_u W_x$ preparation shows slightly greater hydrolytic activity.

TABLE 10

Amounts of glucose formed from waxy starch paste under the action of $s_u W_z$ and $s_u w_z$ pollen extracts. Test 17.

TIME IN HOURS	PERCENT GLUCOSE ON STARCH	
	NON-WAXY EXTRACT	WAXY EXTRACT
0.33	20.48	19.45
8	25.94	22.49
24	28.20	22.48
48	31.56	23.20
72	32.71	23.77
96	34.15	24.21

TABLE 11

Amounts of glucose formed from potato starch paste under the action of $s_u W_z$ and $s_u w_z$ pollen extracts. Test 21.

TIME IN HOURS	PERCENT GLUCOSE IN SOLUTION	
	NON-WAXY EXTRACT	WAXY EXTRACT
0.5	29.59	27.00
6.5	35.21	32.90
28	40.40	33.93
68	42.57	34.50
96	43.70	35.07
125	44.89	35.63

The results obtained with waxy and non-waxy pollen from homozygous sugary plants are very similar, therefore, to those afforded by the corresponding classes of non-sugary pollen. In both series a somewhat higher rate of hydrolysis is consistently shown by the non-waxy pollen. These tests do not indicate, however, that the difference in diastatic activity is increased by the sugary gene.

DISCUSSION

The differential effect of the waxy gene appears to be confined to endosperm and gametophyte. In all other parts of the plant, including the embryo, the granules give the typical starch reaction with iodine. It is not surprising, therefore, to find no difference in the amylase activity of waxy and non-waxy seedlings. The latter contain a large amount of enzyme, which appears to be secreted by cells of the embryo adjacent to the endosperm. These same cells, whether $W_z W_z$ or $w_z w_z$ in composition, lay down an abundance of blue-staining starch grains during

the early development of the seedling. A differential action of the waxy gene is not manifested in this part in the starch formed nor in the amylase accompanying it.

With the object in view of obtaining some light on the character of the amylase concerned in starch synthesis in the endosperm where the waxy gene does exert its action studies were made with water extracts of mature waxy and non-waxy seeds. Tests of the extracts on different starch paste substrates showed that the waxy preparations possessed a significantly higher diastatic activity. Such experiments afford valid evidence on the point in question only if no differential secondary changes affecting the activity of the enzyme in the two classes of seed occur subsequent to the deposition of the starch.

There is good reason for believing that during the period of ripening and storage changes do take place in the seed, particularly in the endosperm, which affect the activity of amylase present. The marked difference in acidity in non-waxy and waxy endosperms which ABEGG's studies reveal is strongly suggestive of secondary reactions which well may alter the amylase. The acid value of the ether extract of freshly ground waxy endosperm meal is much higher than that of non-waxy meal. With age the acidity increases and at a higher rate in the waxy sample. We have observed that the diastatic powers of these meals likewise increase somewhat with age, the change being slightly greater in the waxy material. A definite conclusion that changes in acidity occasion alterations in enzymatic activity is not warranted but the facts make it very doubtful if the results of the tests on seed amylase can be considered a fair measure of the action of the waxy gene on the initial production of this enzyme.

The results obtained with the amylase extracts of waxy and non-waxy pollen are less open to criticism on the grounds that subsidiary changes during ripening and storage may have obscured any difference in the character of the amylase formed during starch synthesis. Starch is laid down in the pollen during a comparatively brief period just previous to anthesis so that material collected as soon as it is shed and dried at once, an operation which kills it, may be preserved in a condition affording little opportunity for modification of the enzymes present. The comparatively simple undifferentiated condition of the pollen would seem to argue for the presence of fewer variables which might influence the factor it is desired to measure. ABEGG has obtained positive evidence that the ether extracts of waxy and non-waxy pollen grains are alike in acidity.

Measurement of the diastatic power of the aqueous extracts of the two kinds of pollen from comparable plants shows that the non-waxy class is more active than the waxy. There is some question in the earliest stages of conversion as to the relative rates of glucose formation due to the difficulty in accurately titrating the rather turbid samples obtained. In some tests the waxy extract appears slightly more active at the beginning and in others the reverse is the case. The differences, however, are within the limits of accuracy of the method employed. As soon as clear readings become possible larger amounts of glucose are invariably found in the samples treated with the non-waxy extract. From about five hours onward to the end of the conversion (96 to 125 hours) the curves clearly show a higher rate of hydrolysis by the non-waxy amylase.

This result is in conformity with the facts we have on the comparative effectiveness of waxy and non-waxy pollen in accomplishing fertilization in the plant. In matings of the types $w_x w_x$ ♀ $\times W_x w_x$ ♂ and $W_x w_x$ selfed, the proportion of waxy seeds is usually slightly in defect of that expected on the Mendelian basis. The fact that the percentage of waxy seeds is the same in the upper and lower halves of the ear, within the limits of random sampling, led us to the conclusion that the deficiency is due to a difference in rate of development during the early stages of pollen-tube growth (BRINK and BURNHAM 1927). It was suggested that the two classes of tubes elongate at different rates so long as they are dependent upon the limited pollen reserves and thereafter at the same rate.

The carbohydrate reserves in the pollen consist largely of starch; those in the style are sugars. It is an interesting and suggestive fact that the waxy gene which, as we have shown, exerts a differential action on the enzyme amylase, does not appear to retard the growth of pollen tubes while the latter are dependent upon the food materials in the style.

The exaggerating effect of the sugary gene (s_u) when in homozygous condition on the waxy deficiency (BRINK and BURNHAM 1927) does not appear to be reflected in the amylase activity of $s_u W_x$ and $s_u w_x$ pollen. The influence of this gene may be exerted on some other factor affecting the initial rate of pollen-tube growth or our method of measuring the diastatic power of the pollen may not be fine enough to detect its action on the enzyme.

Direct tests to determine whether the amylase extracts of waxy or non-waxy pollen hydrolyze the different starch substrates used with equal ease have not been made. The comparison of results obtained on one kind of substrate at one time with those afforded by the other kind of substrate in another trial is not a fair one. Small differences in concentration of

substrate and particularly variations in temperature from one experiment to another may obscure any basic difference in activity of the enzymes. As pointed out earlier, (BRINK 1928) careful tests show that waxy starch is hydrolyzed somewhat less rapidly by malt amylase than is common maize starch. The difference is small but consistent. It appears probable that a difference of the same order would be found with the pollen enzymes. Whatever the relation may be when a given enzyme preparation is tested on more than one kind of substrate the present experiments show that the non-waxy pollen diastase is more active than the waxy on all three kinds of substrates.

The facts afforded by studies on pollen-tube growth and on the character of the pollen reserves and the enzymes accompanying them appear to be best accounted for on the assumption that the primary effect of the waxy gene is a modification of the kind or amount of amylase produced. It might be argued that the waxy gene is not directly concerned in diastase formation at all but acts upon some other process or processes whose net effect is a lowered diastatic activity. We have no evidence invalidating such an hypothesis. There are circumstances surrounding the action of the waxy factor, however, which favor the conclusion that relation between gene and enzyme is a direct one. The evidence relates to the manner in which the waxy factors operate to modify rate of pollen-tube growth.

Let us compare briefly the conditions under which the sugary and the waxy genes influence the development of the male gametophyte as shown by their effect on the ratio of waxy seeds on segregating ears. A consideration of this evidence makes it apparent that the waxy gene operates in a direct way whereas the sugary factor exerts its influence in some roundabout fashion. $W_z w_z$ parents selfed or used as staminate parents in the backcross to the $w_z w_z$ type produce a small deficiency of waxy seeds amounting to about one and two percent, respectively. If the waxy heterozygotes are homozygous for the sugary gene ($s_u s_u W_z w_z$) the deficiency is much larger (roughly 15 percent in the backcross). It is a highly significant fact that when the double heterozygote ($S_u s_u W_z w_z$) is used as the pollen parent in combination with the double recessive type ($s_u s_u w_z w_z$) the large deficiency of waxy seeds in the sugary class is not in evidence. The sugary and non-sugary groups both contain about two percent fewer waxy seeds than expected on the 1:1 basis. The two classes of pollen, $s_u W_z$ and $s_u w_z$, therefore, behave differently according to the type of sporophyte producing them. Only when the

diploid plant is homozygous for sugary do we find an effect on the pollen leading to a large deficiency of waxy seeds.

This behavior is accounted for on the assumption that under the sustained action of the sugary gene in homozygous condition the cytoplasm, or some constituent of it such as the plastids, is modified during the development of the sporophyte. At sporogenesis the modified cytoplasm is passed on to the gametophytes. Waxy gametophytes possessing the "sugary" cytoplasm are handicapped to a much greater extent than the alternative non-waxy class. In $S_u s_u W_z w_z$ plants, on the other hand, no change in the character of the cytoplasm occurs on account of the presence of the dominant non-sugary gene, S_u. According to this hypothesis, then, the sugary gene modifies the character of the pollen indirectly through its previous action on the cytoplasm of the sporophyte.

In contrast with the behavior of the sugary gene, the waxy factor shows its effect in the gametophyte generation immediately after separation from its dominant allelomorph. In the character of the starch laid down in the pollen (and in the embryo sac also) we have a direct response to the presence of the waxy gene. Waxy and non-waxy microspores arising from the same diploid plant at once become differentiated in their carbohydrate metabolism. This behavior is intelligible if we assume that the waxy gene instead of acting on the development of some cell organ, as the sugary factor appears to do, controls the production of an enzyme which functions at once in starch synthesis.

ACKNOWLEDGMENTS

The writer desires to acknowledge the assistance of AUGUSTIN RODOLFO in making the glucose determinations reported here. The work was carried out in part under a grant from the Research Fund of the UNIVERSITY OF WISCONSIN.

SUMMARY

1. A series of experiments designed to obtain a measure of the activity of the enzyme amylase produced under the action of the waxy gene in pollen, seeds and seedlings has been made. The conditions of the tests were arranged as simply as possible. The material was ground and treated with water and the crude extract tested directly on substrates of potato, waxy maize or non-waxy maize starch pastes. Hydrolysis was measured by the amount of glucose formed.

2. Seven tests with the aqueous extracts of waxy and non-waxy pollen from comparable plants are in agreement in showing a significantly greater hydrolytic activity of the non-waxy material.

3. The difference between the enzymatic activity of pollen from $s_u s_u W_x W_x$ and $s_u s_u w_x w_x$ plants is apparently not greater than that between pollen from $S_u S_u W_x W_x$ and $S_u S_u w_x w_x$ plants. It is known that the sugary gene may affect the ability of waxy and non-waxy pollen to grow in the style of the plant but this difference does not appear to be reflected in diastatic power.

4. Results which appear at first contradictory to those mentioned above were obtained with the extracts of seeds from segregating ears. Here the waxy preparation showed the greater starch hydrolysing power. It is pointed out, however, that there is a strong likelihood that secondary changes occur in the seed during ripening and storage which considerably modify its diastase content. The reasons for considering that the results with pollen afford a much more accurate picture of the effect of the waxy gene on amylase production are discussed in the text.

5. Water extracts from young waxy and non-waxy seedlings show equal diastatic activity. This is to be expected in view of the fact that the embryo cells from which this enzyme largely comes form blue-staining starch grains whether $w_x w_x$ or $W_x W_x$ in composition.

6. The facts previously published on the effect of the waxy and sugary genes on pollen-tube growth are briefly reviewed. This evidence points to the conclusion that the action of the waxy factor on starch synthesis is a direct one. In other words, the chain of physiological processes linking the gene and its end result is, comparatively speaking, short and simple.

7. The evidence from pollen-tube studies and from the present biochemical experiments seems best accounted for on the assumption that the waxy gene primarily alters the kind or amount of amylase formed in those parts where reserve starch is laid down. The results with pollen extracts show that the waxy factor lowers the diastatic activity of the enzyme. The change may be one of amount or may be qualitative in nature.

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A CYTOLOGICAL STUDY OF MEIOSIS IN THE POLLEN MOTHER CELLS OF SOME OENOTHERAS[1]

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INTRODUCTION

The genetical work on Oenothera which resulted in the Mutation Theory of DE VRIES dates back to 1885. As DE VRIES introduced the method of strictly controlled self-fertilization in his breeding of the Oenotheras he felt safe in regarding the new forms he discovered as mutants rather than hybrids. Some critics have regarded *Oenothera Lamarckiana* as a hybrid, and have assumed that the new and unexpected types which appear in each generation are the result of segregation. For those who shared the latter opinion there remained the question as to why the heterozygous plant breeds true with only the rare appearance in large numbers of these unexpected segregates, unlike the usual behavior in other organisms which are hybrids. This so-called "Oenothera problem" has been well stated by SHULL (1923a) in this way: "Why is it that with chromosomes visibly behaving as they do in other organisms, extensive and intensive breeding experiments covering nearly thirty-

[1] Financial assistance in the form of a CHINA MEDICAL BOARD Fellowship and the FRANCIS HINTON MAULE Fellowship in Biology of PRINCETON made possible the beginning of this study in 1923 at PRINCETON UNIVERSITY and its completion at the UNIVERSITY OF NANKING.

five years have led many able geneticists to the view that Oenothera is genetically a law unto itself"? The answer given by SHULL is that with the exception of the short styles of *brevistylis* and several other characters (*vetaurea*, *supplena* and *bullata*) recently discovered, all other characters thus far studied, by which Oenothera species are distinguished, are determined by factors which appear to be closely associated in a single linkage group.

In the latter part of 1917, MULLER published his first paper on the subject of balanced lethals, in which he suggested that, due to its close analogy to the case of beaded wings in Drosophila, balanced lethals in Oenothera "probably lie at the root of nearly all the unusual genetic phenomena of the genus." MULLER found the beaded wing condition to be lethal in effect when in the homozygous condition, and the reason why the beaded condition did not breed true was because each beaded individual was heterozygous for the normal allelomorph of beaded. He later found another lethal associated with the normal allelomorph of beaded.

Crossing over between this new lethal and beaded would bring about a condition whereby the normal characteristic might find expression. If a recessive mutant gene should appear in one of the "balanced" chromosomes and then if a crossover between this recessive mutant gene and the lethal factor should occur, there would be released a mutant factor which, when homozygous, could find expression. Several of the Oenothera geneticists had already arrived at essentially the same conception, though not using the expression "balanced lethals." DE VRIES (1907) had produced his *laeta-velutina* twin hybrids from crosses made between *Oe. biennis* or *Oe. muricata* (*syrticola*) and *Oe. Lamarckiana* or some of its mutants. RENNER (1914, 1917a), in attempting to explain this peculiar behavior, found that about half the pollen of *Lamarckiana* failed to germinate. He supposed that *Lamarckiana* was a permanent heterozygote for the gene complexes, *gaudens* and *velans*. The gene-complex *gaudens* produces the twin *laeta*, while the gene-complex *velans* produces the twin *velutina*. He also assumed that the homozygous conditions, as *gaudens-gaudens* and *velans-velans*, were lethal in effect and caused the non-viability of the seeds that failed to germinate. DE VRIES (1916, 1918) also adopted this view. Exchange of materials between the complexes *gaudens* and *velans*, RENNER also suggested, gives rise to certain of the mutant types.

In addition to the work just referred to, RENNER has carried on intensive studies on five Oenothera species, *Lamarckiana*, *biennis*, *muricata*,

suaveolens, and *Hookeri*, and their hybrids, with the idea of attempting to clear up other Oenothera peculiarities. In his paper of 1925 he summarizes his analyses of the gene complexes of these five species. His chief emphasis has been on the gene complexes themselves, to which he has given special names, rather than upon the species which the complexes in part represent. Practically all of the possible combinations of these eight gene complexes have been obtained and their offspring studied, but no data are offered to show how these complexes are related in terms of genes.

In reference to the interchange of factors between these gene complexes, RENNER offers several possible interpretations. First, he considers it quite unlikely that the factors involved are all linked in one chromosome pair, since some factors have been found linked in some gene-complex combinations while in others they are completely independent. He adds, "Typical factor linkage with occasional crossing over has been observed in a factor group, which corresponds to chromosome I of SHULL." Again, he suggests that it might be assumed that all these genes are really independent in inheritance. In this connection he has found that many of the new factor combinations appear to be non-viable, which perhaps explains the many empty pollen grains, although the loss of gametes and zygotes in this manner would not be sufficient to account for all the different heterozygous genes. He thus would assume that the parental combinations are more numerous than most of the new combinations. Such persistence of parental combinations is held to support the supposition that there is a kind of selective chromosome coupling in the reduction division. This chromosome coupling or "linkage," as suggested by CLELAND, does not permit any random assortment of the chromosomes at the reduction division. With this idea of chromosome linkage in mind, RENNER finally has attempted to arrange the genes, separated from the various complexes, among five of the seven chromosomes.

In 1915 and 1916 SHULL (1923a, p. 89) recognized the first undoubted cases of crossing over in Oenothera, which he had suspected in 1913. Professor SHULL first published on this subject in 1921, concluding that (p. 216), "On the whole it is now clear that while the genetical phenomena in the Oenotheras, with exception of the case of variegated foliage, can be referred definitely to the chromosomes, the occurrence of independent segregation which is necessary for the production of typical Mendelian behavior is so rare as to be almost negligible."

In a paper read before the SECOND INTERNATIONAL CONGRESS OF EUGENICS in New York in 1921, SHULL (1923a) presented genetical evidences of linkage with crossing over in *rubricalyx* buds and red stems, *rubricalyx*

buds and *nanella* stature, pink-coned buds and *nanella* stature, and linkage of revolute leaves with *rubricalyx* buds. The details of this evidence are found on pages 89–93. In the same paper, he assumed the occurrence of gamete and zygote lethals, in addition to the fact of linkage with crossing over, to explain "the complex nature of the segregation." Another possible explanation which SHULL (p. 94) had previously favored was "the nuclear-chimera idea that chromosomes were hanging together in clusters," and other workers had suggested "that there were so many non-compatible combinations that only a small number, represented by the combinations which actually appeared, were capable of existence." The nuclear-chimera hypothesis involves the idea of groups or chains of chromosomes, which in fact have since been recognized by CLELAND (1922), HÅKANSSON (1926) and others to be rather constant features in the nuclear behavior of the species, crosses and mutants thus far studied. This idea and its possible significance, as well as the non-compatible combinations of factors as applied to entire chromosomes, will be discussed more fully subsequently in this paper.

In 1922, GATES, following SHULL'S statement of the previous year (published 1923a), announced that the cytological study of the Oenotheras made him conclude that they were not favorable to the idea of crossing over. This conclusion was based upon the fact that the Oenothera chromosomes are arranged telosynaptically like a string of sausages and by the time they arrive at diakinesis they are already in so short and stout a condition that twisting about each other would be impossible. He says, "This is a very disturbing fact for those who write about 'crossing over in Oenothera'."

In reply to GATES, WHITING (1923) pointed out that crossing over does not take place between fully developed chromosomes but rather during early stages when the chromosomes are in a long threadlike condition, as shown by PLOUGH (1917) in his temperature experiments on Drosophila.

SHULL (1923b) later advanced further genetical evidence for crossing over in the Oenotheras. In this connection he says (p. 166), "The cytological studies of Oenothera have thus far failed to discover a relation between the homologous chromosomes which seems favorable to the assumption that an interchange of genes between them may take place by the method of chiasmatypy. This fact must not be allowed, however, to throw any doubt on the occurrence of crossing over in this group, as demonstrated by appropriate breeding experiments."

It was with some of these facts before us that Professor CONKLIN suggested that a cytological study be made on the pollen mother cells in

order to secure some cytological evidence bearing on the genetical conclusions of Professor SHULL.

I wish to thank Professor G. H. SHULL for his interest in this study and many kind criticisms and suggestions offered, as well as for the seeds which he kindly gave me and which were planted at Nanking, China, where most of the work was done during 1925 and 1926. For the many suggestions and his interest shown throughout the investigation, I also wish to express my thanks to Professor E. G. CONKLIN.

MATERIAL AND METHODS

The Oenotheras used in this study were collected during three consecutive summers. The first lot was collected during the late summer and early fall of 1923, from the Oenothera experimental gardens of Professor G. H. SHULL, Princeton, New Jersey. Due to an unusually severe drought during the summer, some of these plants unfortunately failed to provide adequate material. Those which I have studied from this collection and which will be reported on here, consisted of *Oenothera Lamarckiana*, *Oenothera* mut. *pervirens*, and *Oenothera rubricalyx sulfurea*.

The second collection was made during the summer of 1924 from the same experimental garden. They were fixed and taken to China in 80 percent alcohol for further study. These consisted of the following species, mutants, hybrids and segregates: *Oe. grandiflora* B, *Oe.* seg. *deserens*, *Oe. franciscana sulfurea*, *Oe. suaveolens* (yellow), *Oe. suaveolens* (sulfur), *Oe.* (seg. *decipiens* × *grandiflora*) F_1, *Oe.* (seg. *decipiens* × *grandiflora*) F_2, *Oe.* (*grandiflora* × seg. *decipiens*) F_2, *Oe. pratincola*, *Oe.* mut. *pervirens*, and *Oe. Lamarckiana*.

In the spring of 1925, seeds which had been given to me by Professor SHULL were planted in the Agricultural Gardens of the UNIVERSITY OF NANKING. From these I was able to secure buds from *Oe.* (seg. *decipiens* × *grandiflora*) F_1 and *Oe.* (*grandiflora* × seg. *decipiens*) F_2.

At least six different collections were made in each case between 10 and 12 o'clock in the morning, covering a period of several weeks. Buds were selected not from one plant alone but from a large number of plants of each form. The 1923 collection was fixed immediately afterward in the laboratory. The 1924 and 1925 collections were fixed partly in the field (in the case of BOUIN'S modified mixture) and partly in the laboratory (in the case of CARNOY-LEBRUN'S solution).

The following fixing fluids were employed: Strong FLEMMING, a modified FLEMMING as used by Doctor TAYLOR of the UNIVERSITY OF PENN-

SYLVANIA, BOUIN'S picro-formal-acetic and BOUIN'S modified according to MCCLUNG, CARNOY-LEBRUN'S equal parts chloroform, absolute alcohol and acetic acid, saturated with corrosive sublimate. These were all freshly made up before using.

Some buds were first dipped in chloroform for an instant, before placing them in strong FLEMMING or its modification, in order to remove the waxy substance on the stamens and thus insure faster and more uniform penetration. The loculi of the anthers of a few were split open before placing them in the fixing fluids. One-percent maltose was added sometimes to each fixative of FLEMMING'S or its modified formula, in order to prevent excessive clumping of the chromosomes. The value of this latter procedure cannot be vouched for, since equally good results were obtained when this was not added. The time of fixation for these two fluids was from ten hours to three days. For BOUIN'S and BOUIN'S modified, no preliminary chloroform washing was employed. These were fixed for periods of from one to three days. CARNOY-LEBRUN'S mixture was used mostly for periods of ten to fifteen minutes.

Smears were made in several instances using modified FLEMMING and modified BOUIN'S, giving in some cases splendid results.

The best results came from CARNOY-LEBRUN'S solution and from modified BOUIN'S, especially the latter. The usual steps following the respective fixing fluids were employed. In each case very gradual changes were employed so as to insure as nearly natural conditions as possible.

All buds were imbedded in paraffin and cut from 5 to 8 microns in thickness, most of them being 7 to 8 microns. In each instance sections and smears were stained with HAIDENHAIN'S iron-alum haematoxylin.

DESCRIPTION

The cytological structure of the early stages of development of the pollen mother cells is so similar in the twelve different forms studied that the description for these stages will be based largely upon *Oe. Lamarckiana*. These early stages will include those from the archesporial cells to the latter part of the heterotypic prophase, and again from interkinesis to the telophase of the homotypic division. In a few instances figures will be shown of some of these stages as seen in some of the other forms.

The term synizesis will be used throughout this study to cover the two contraction periods through which the nuclear reticulum passes during the heterotypic prophase. The first synizetic period results in a ball-like mass of chromatin threads called the first contraction. The second syni-

zetic period, following an open spireme stage, results in the second contraction.

All measurements given for any particular stage are averages made on at least six different cells. These measurements were made with a 7.5 Bausch and Lomb ocular micrometer using a 1.9 oil immersion objective on a Bausch and Lomb microscope with tube length 190.

Archesporium

The archesporial stages are found in anthers about 2 mm or less in length. The pollen mother cells lie arranged in one or two rows between tapetal cells, very tightly fitted together. The earliest stages that were found, corresponding to a late telophase of the last archesporial division (figure 19), show the nucleus to be small and surrounded by considerable cytoplasm. Distributed through the nucleus or arranged peripherally are definite chromatic bodies or "prochromosomes" lying among a mass of fine, apparently anastomosing linin fibers (figure 1). The nucleolus is a rounded black structure lying to one side in the nucleus. Measurements made show the nucleus to be 4.5μ and the nucleolus 2.04μ in diameter. During the growth period which follows, these chromatic bodies finally disappear as definite structures, and in later stages of the archesporial cell they appear as clumps of chromatin material connected with fine anastomosing reticular threads (figure 2). At this time the nucleolus has increased slightly in size, 2.04μ to 3.06μ in diameter. The nucleus also has grown from 4.5μ to 6.12μ in diameter. Finally the nucleus becomes filled with a dense network of fine threads in which are still seen some small chromatin particles or knots, the karyosomes, which are quite evident at the junction of the threads (figure 3). In approximately this condition the pollen mother cell passes through a relatively long resting period which immediately precedes the advent of the first synizetic period. The reticular threads may be finer and more numerous than shown in figure 3. This long resting period is evidenced by the fact that these stages are numerous and easily found.

At no time during this growth and rest period in the twelve forms studied was there any evidence among the threads of any significant parallelism that could be attributed to a longitudinal splitting, later obscured to form a univalent spireme, as noted in Osmunda by Miss DIGBY (1919, page 139); neither was there any indication of parallelism and synapsis of univalent threads. The fineness of the threads and in most cases their faintly visible structures, make any definite conclusions in favor of either contention impossible.

Description of Plates

Figures 1 to 4, 6 to 11, 15, 16, and 23 to 26 were drawn with a Leitz microscope, 1/12 oil immersion, number 4 ocular, tube length 190 mm at table level with a Zeiss camera lucida. The approximate magnification at which they were drawn was 2300×. All the other figures were drawn with a Bausch & Lomb microscope, 1.9 oil immersion, number 15 ocular, tube length 190 mm at table level with a Bausch & Lomb camera lucida, with an approximate magnification of 2700×.

Plate 1

Oenothera Lamarckiana

Figures 1, 2, 3.—Three successive stages of the archesporial cell.

Figure 4.—Smear preparation showing early synizesis.

Figure 5.—Early synizesis.

Figures 6, 7.—Later stages in synizesis.

Figure 8.—First contraction complete.

Figure 9.—Opening of the first contraction stage.

Figure 10.—Open spireme.

Figure 11.—Second contraction complete.

Figure 12.—Opening of the second contraction; individual chromosomes evident for the first time as a result of constrictions in the spireme.

Figure 13.—Early period of late heterotypic prophase showing 1 pair and a circle of 12 chromosomes.

Figure 14.—Later period than figure 13. The chromosomes have shortened. One chromosome of the pair does not show in the section.

Figures 15, 16.—Smear preparations showing early anaphase. Regularity is evident in the movement of the chromosomes to their respective poles.

Figures 17, 18.—Mid and late interkinesis respectively.

Figure 19.—Homotypic telophase.

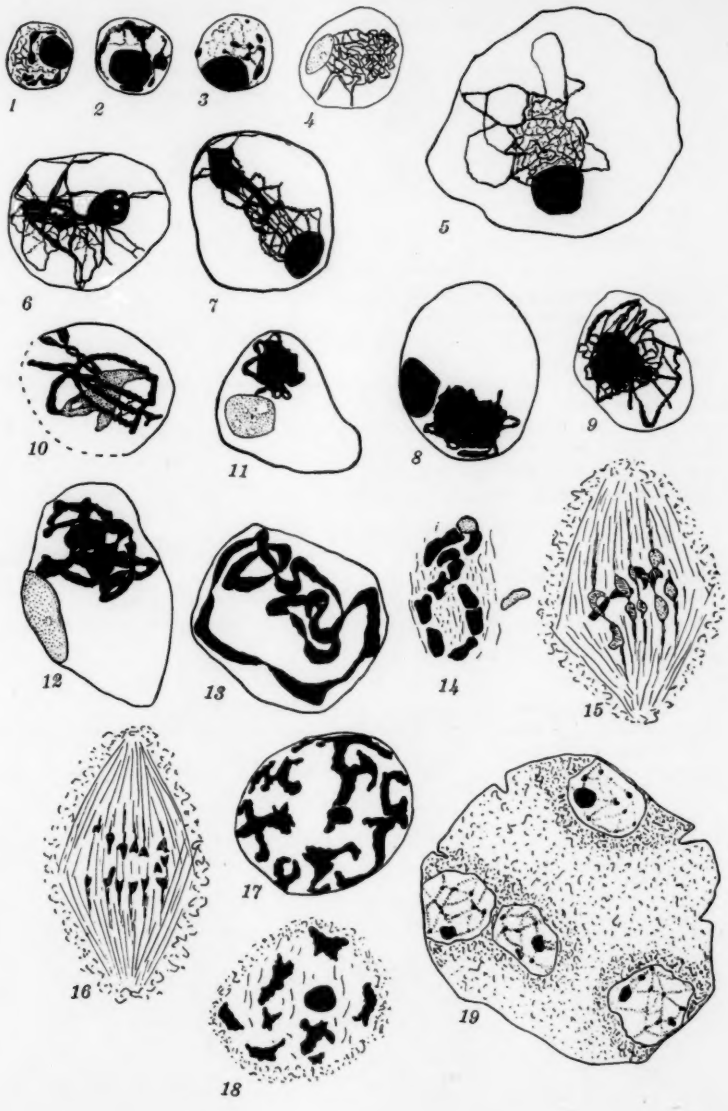
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PLATE 2

Oenothera mut. *pervirens*

FIGURE 20.—Heterotypic prophase showing 1 pair and a closed circle of 12 chromosomes. This arrangement was the more common.

FIGURE 21.—Same stage as figure 20 but showing another chromosome arrangement, 7 complete pairs.

FIGURE 22.—Heterotypic metaphase. Arrangement of the chromosomes is regular. Some adjacent chromosomes of the circle still united.

FIGURE 23.—Late heterotypic telophase or interkinesis.

FIGURE 24.—Tripolar homotypic prophase.

FIGURE 25.—Typical homotypic metaphase.

FIGURE 26.—Homotypic telophase in early stage.

Oenothera rubricalyx sulfurea

FIGURE 27.—Typical second contraction showing loops. Twisting and apparent fusions taking place between halves of individual loops.

FIGURE 28.—Late heterotypic prophase showing 7 individual chromosome pairs.

FIGURE 29.—Slightly later period when the spindle fibers are being laid down and the 7 pairs are arranging themselves on the spindle.

Oenothera grandiflora B

FIGURE 30.—Open spireme. Some threads not shown.

FIGURE 31.—Typical second contraction showing loops. Twisting and apparent fusions of the sides of some of these loops.

FIGURE 32.—Late heterotypic prophase showing 7 individual pairs with spindle fibers forming.

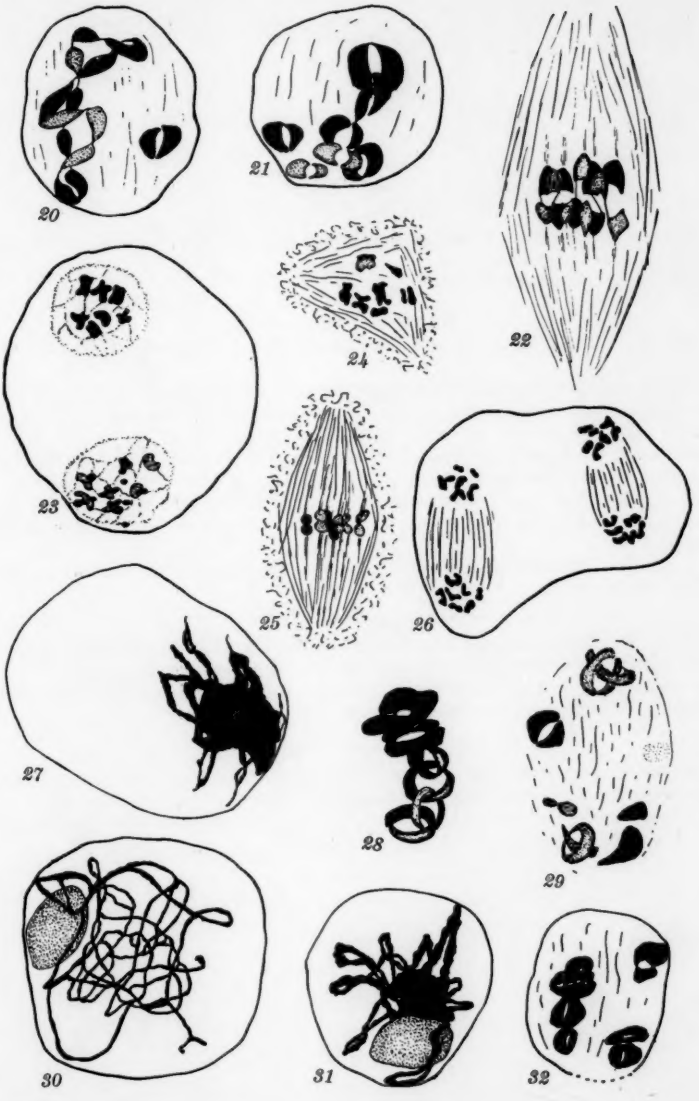
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PLATE 3

Oenothera grandiflora B

FIGURE 33.—Typical heterotypic metaphase of *grandiflora*, showing some premature breaking of one end of the chromosome pairs.

FIGURE 34.—Heterotypic telophase.

Oenothera franciscana sulfurea

FIGURE 35.—Loosening of the second contraction and the formation of the heterotypic chromosomes.

FIGURES 36, 37.—Same stage as figure 35. Twisting is still evident and in figure 37 one pair of chromosomes shows a remnant of what was possibly a former connection between the two members of the pair.

FIGURE 38.—Heterotypic prophase. Five complete pairs are present while the other two pairs are incomplete in this section.

FIGURE 39.—Heterotypic prophase also. Here six complete chromosome pairs appear in synapsis. The seventh pair is not present in this section. Chromosomes are still in an extended condition.

FIGURE 40.—Late heterotypic anaphase showing great regularity of chromosome distribution to their respective poles.

Oenothera suaveolens (yellow)

FIGURE 41.—Late heterotypic prophase. Spindle fibers are forming and the one pair and closed circle of twelve chromosomes are arranging themselves on the spindle.

FIGURE 42.—Heterotypic metaphase. There are indications that adjacent chromosomes are migrating to opposite poles in a regular fashion.

Oenothera suaveolens (sulfur)

FIGURES 43, 44.—Heterotypic prophase. Both sections show 1 pair and a closed circle of 12 chromosomes. The chromosomes are still slightly elongated.

FIGURE 45.—Early heterotypic anaphase. Adjacent chromosomes passing to their respective poles in a zigzag manner.

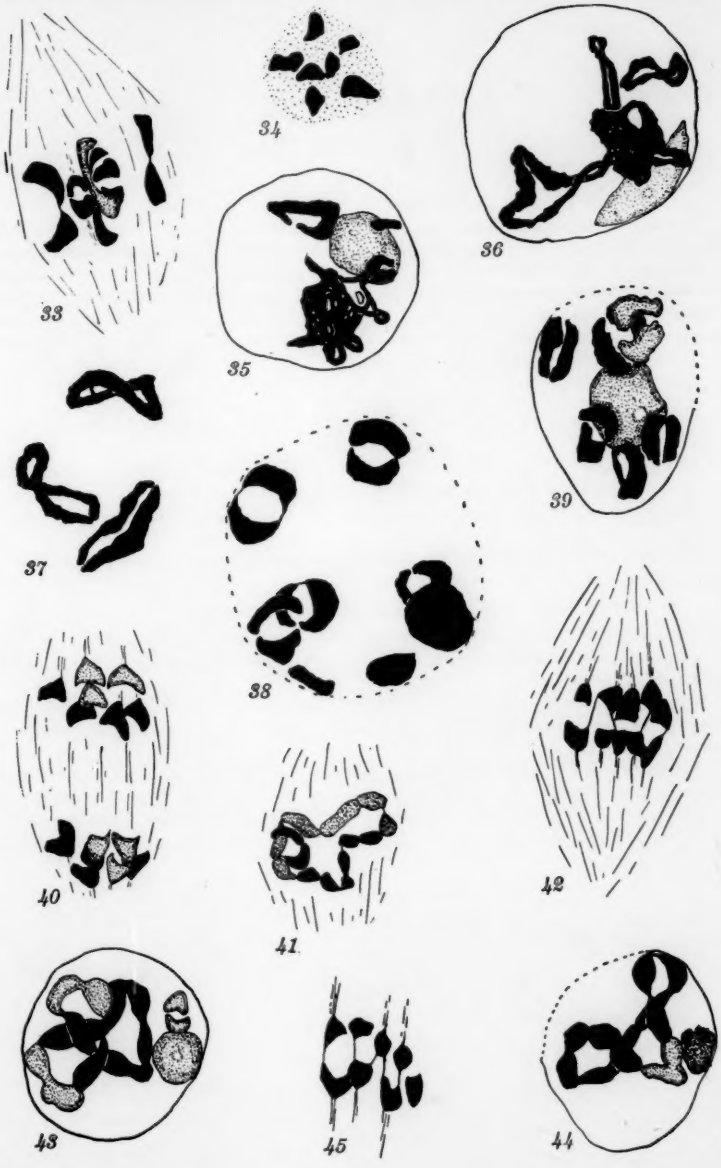
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PLATE 4

Oenothera pratincola

FIGURE 46.—Heterotypic prophase. One pair and a very twisted closed circle of twelve chromosomes. Nucleolus is very light brown due doubtless to its loss of chromatin material.

FIGURES 47, 48.—Heterotypic metaphase or late prophase. Some of the chromosomes of the closed circle are arranging themselves regularly and apparently effecting an equal numerical distribution by adjacent chromosomes passing to opposite poles. In figure 47 several chromosomes are omitted for the sake of clearness. In figure 48 the one chromosome pair to complete the closed circle was not present in this section.

Oenothera seg. *deserens*

FIGURE 49.—Loosening of the second contraction stage showing the formation of diakinetic pairs.

FIGURES 50, 51.—Heterotypic prophase. Figure 50 shows a closed circle of 6 and 3 pairs, while figure 51 shows a closed circle of 6 and 1 pair. The closed circle of 6 was very constantly seen.

FIGURE 52.—Polar view of early heterotypic anaphase. The haploid chromosome number present.

FIGURE 53.—Mid-interkinesis. The seven chromosome pairs are split and attached back to back or slightly at one end.

Oenothera (seg. *decipiens*×*grandiflora*)F_1

FIGURE 54.—Smear preparation of an open spireme stage. A few threads are not shown.

FIGURE 55.—Heterotypic prophase. A closed circle of 6 chromosomes and 4 pairs. Chromosomes are still in an elongated condition.

FIGURE 56.—Late heterotypic prophase with bipolar spindle and chromosomes arranging themselves on the spindle in such a way that apparently adjacent chromosomes will pass to opposite poles.

FIGURE 57.—Heterotypic metaphase. Chromosomes regularly arranged and with some connections visible which would indicate that adjacent chromosomes will pass to opposite poles.

FIGURE 58.—Mid-interkinesis. The seven splitting homotypic chromosomes present with six of them arranged peripherally, the seventh centrally located. This is a very common arrangement.

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PLATE 5

Oe. (seg. *decipiens*×*grandiflora*)F_2

FIGURE 59.—Open spireme stage. A few threads not shown. Spireme thread more or less uniform in size.

FIGURE 60.—Second contraction under way, evidently beginning at the center of the mass.

FIGURE 61.—Heterotypic prophase with 7 individual pairs of chromosomes.

FIGURE 62.—Late heterotypic prophase showing the paired chromosomes arranging themselves on the spindle. One chromosome of one pair had been evidently pushed out of the spindle by the knife in cutting.

Oe. (*grandiflora*×seg. *decipiens*)F_2

FIGURE 63.—The spireme entering second contraction.

FIGURE 64.—Complete second contraction showing two loops which evidently are composed of chromosome pairs uniting in synapsis.

FIGURE 65.—Heterotypic prophase. Six chromosomes in a circle and 4 diakinetic pairs.

FIGURES 66, 67.—Heterotypic prophase showing another arrangement of the chromosomes which is the more common one, seven individual pairs.

FIGURE 68.—Late heterotypic prophase showing the six chromosomes of the closed circle arranging themselves on the spindle.

FIGURE 69.—Heterotypic metaphase showing what were evidently seven chromosome pairs in diakinesis, arranging themselves on the spindle.

Oe. (*grandiflora*×seg. *decipiens*)F_2 (raised in China)

FIGURE 70.—Smear preparation of heterotypic prophase with 4 individual pairs and a closed circle of 6 chromosomes. The nuclear membrane is dissolving away.

FIGURE 71.—Late heterotypic prophase. The 4 chromosome pairs and the closed circle of 6 arranging themselves on the spindle. The nucleoli in figures 70 and 71 are fast disappearing.

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Early heterotypic prophase

The entire heterotypic prophase, from the first appearance of contraction of the fine network of reticular threads immediately following the archesporial resting stage until the final prophase stage, is in general one of the most difficult to interpret as well as one of the most significant periods in the development of the pollen mother cells. It is also the longest stage through which the pollen mother cells pass during their development.

Synizesis

This period sets in immediately after the close of the resting period of the archesporial cell by a process of gradual condensation of the chromatin material and contraction of the fine reticular network of threads. The first stage of synizesis shows the nucleus containing a dense mass of fine threads, oftentimes slightly beaded. The nucleolus lies to one side of the center of the nucleus in contact with the reticular mass of threads, but not necessarily in contact with the nuclear membrane (figures 4, 5).

The beaded condition which is usually seen at this time, and the gradual unequal thickening of some of the threads, are strong indications that condensation of the chromatin material is taking place at intervals along the reticular network of threads. There are no indications that fusions occur among the threads at this time resulting in a decrease in their number. From measurements made, the nucleus has increased to about twice its former diameter. The nucleus of figure 5 measures 12.3μ and the nucleolus 3.06μ.

Movements of the nucleolus and reticular threads accompany the increase in nuclear size. All of these changes are the general result of cell growth, which is rapid at this time. If we assume that the increase in karyolymph during the nuclear increase in size is due to penetration of cell sap into the nucleus, we might conclude that the nuclear and reticular thread movements are due to pressure exerted upon them by the incoming cell sap. Whatever the factors involved, numerous observations and measurements show that there is a large nuclear growth accompanied by movements of the nucleolus and the reticular threads toward one side of the nuclear cavity. The nucleolus and reticular threads may finally occupy any side of the nucleus.

It is impossible to determine any individuality of the threads, or any splitting of them. Except for what would be expected in any such apparently tangled mass of threads, there is apparent no parallelism which might

be interpreted as parasynapsis. From all appearances the mass of threads, at this time as well as later, is a continuous thread rather than many individual ones. This is partly indicated by the numerous loops that can be seen along the periphery of the mass, both in sections (figure 5) and in smear preparations (figure 4). Further evidence for a continuous spireme is of course necessary and in fact is to be seen at a later stage when the spireme has become thickened and shortened (figure 59).

In later stages of synizesis the nucleus remains approximately the same size, 10.7μ to 13.77μ in diameter. The cell wall has increased from what appears to be a thin line to 3.06μ in thickness. The nucleolus remains about the same size. The changes that take place now involve principally the nucleolus and chromatin threads. These chromatin threads decrease materially in number due to the continued contraction and possibly to fusions of the reticular threads, although, as was true also in the earlier stages of synizesis, no indication of the fusion of any threads was observed. The threads lying adjacent to the nucleolus appear to have fused with it and apparently act as avenues of exit for the chromatin material stored in the nucleolus (figures 6, 7). Such a process would indicate that the linin fibers, as the reticular threads appear to be, constitute the fixed or permanent structure while the chromatin substance flows on, or in, the linin fibers. In this connection, MONTGOMERY (1900, page 352) in his study of *Peripatus balfouri*, says: "I would conclude that most of the movements of the chromatin are phenomena automatic on its part, probably motions of a flowing nature. And here it must be kept in mind that the chromatin is not made up of solid microsomes, but has a more or less thickly fluid consistency, so that a flowing on its part is quite possible." Referring to the chromosomes (page 349) he adds, "the individuality is retained apparently by virtue of the persisting linin connections."

If the permanent structures are the linin threads, and if the chromatin material flows out of the nucleolus on or in the fibers during synizesis, then the genes or inheritance factors must be located in these linin fibers in fixed positions.

If the contraction stages in Oenothera are normal, as there is every reason to believe, the linin threads certainly are contractile. The fact that these early synizetic stages have been observed in buds along with later stages of synizesis, diakinesis and heterotypic metaphase, all in a very well fixed condition, indicates that synizesis is a normal and significant period in the development of the pollen mother cells and not an artifact. This contractility appears to be unequal and not simultaneously

active in all parts of the reticular threads, since some regions show more rapid contraction than others (figures 6, 7). This was always found to be the case. To say that synizesis is not an artifact does not preclude the possibility that slight contractions are due to the fixing fluids. But these play a minor rôle while the factors which produce synizesis play the major rôle.

As the chromatic material passes out from the nucleolus to the linin fibers, the nucleolus often stains a light brown instead of the intense black that characterizes its earlier stages and very often shows different degrees of vacuolization. Vacuolization may occur even though the nucleolus stains an intense black (figure 6). There is also evidence that the chromatin material passes along from one point of the linin fibers to another, since definite thickenings appear at intervals upon staining, producing an irregular beaded condition (figure 7). As this process continues, some of the threads disappear while the others become thicker and more beaded, the beaded structures being arranged in single rows, until finally the whole mass becomes tightly contracted, occupying a peripheral position in the nucleus. This marks the end of the first synizetic period and gives rise to the first contraction stage (figure 8). In this tightly contracted mass no details can be determined except a few small loops on the periphery. From the tangled mass of fine threads of early synizesis, there appears a continuous spireme of a more or less uniform thickness which is best seen during the open spireme stage.

Throughout this entire synizetic period there is no splitting of any kind. All observations thus far reported (GATES, DAVIS, CLELAND and others) indicate a continuous univalent spireme thread.

Open spireme

Following the first contraction stage, the chromatin threads begin to loosen, resulting in numerous loops being thrown out from the central contracted mass (figures 9, 54). The loops are still beaded and exhibit no particularly significant appearance. At the time of its complete opening, the spireme has become relatively uniform in thickness and quite shortened, with very fine threads no longer present (figures 10, 59). A few threads in these cells have been omitted for the sake of clearness. The nucleolus is also usually brown or light brown in color, and lies in the middle or slightly to one side in the nucleus and is no longer pressed tightly against the nuclear membrane. This condition may be evidence that much of the chromatin has already passed out onto the spireme. It is at this time that

the spireme can be easily identified as a continuous structure. The open spireme stage is a relatively short one, in fact just long enough for condensation to play its part in the spireme formation. The contractile nature of the spireme again manifests itself in the formation of a second contraction, beginning apparently in the central region of the spireme mass (figures 11, 27, 31, 64). Synizesis therefore produces a univalent spireme which is later segmented or constricted into the characteristic somatic number of chromosomes in preparation for meiosis. This is a very significant behavior in Oenothera.

Second contraction

In every instance of second contraction, loops may be seen, varying in size and number, projecting from the contracted mass (figures 11, 27, 31, 64). There are indications of close approximation and twisting of the sides of some of these loops, resulting possibly in permanent fusions (figures 27, 31). These stages are not uncommon. The loops apparently are not just ordinary loops of an undifferentiated, contracted and thickened spireme, but rather differentiated loops that represent halves of homologous chromosome pairs in the process of diakinesis. This fact becomes more apparent as the contracted mass begins to open (figures 12, 35, 36). Figure 37 represents this stage where the chromosomes have just been cut off in pairs. They are still greatly elongated with irregular, roughened sides. Besides twisting, one pair also shows a connection between the two halves. Figure 36 shows two pairs cut off and another in the process. Figure 35 shows one pair already cut off from the mass while another is in the process of pairing and being cut off by segmentation. Figure 49 also shows three pairs already cut off and several others forming.

This represents a very critical stage. The chromosomes are in the process of formation by segmentation or constriction of the completed spireme and it is the first time that definite individual chromosomes appear to come into intimate relationship with one another. Their elongated roughened appearance and their twisted and apparently fused condition lead one to recognize that this particular stage is well suited for the exchange of chromosome parts required by the genetical evidence. This observation and conclusion, first made by the writer in 1923, has been confirmed by evidence based on additional material. CLELAND (1926) and HÅKANSSON (1926) have reported similar cases of twisting and apparent fusion of homologous chromosomes, and they likewise suggest that a mechanism

is thus provided for crossing over between factors lying within a pair of homologous chromosomes.

That segmentation takes place at this time, resulting in closed chromosome circles or diakinetic pairs, points strongly to the view that the sides of these loops represent the halves of homologous pairs of chromosomes, presumably of maternal and paternal origin, respectively. The spireme thus appears to be an organized structure, composed of homologous chromosome pairs joined end to end. Cytological proof of their maternal and paternal origin can not be adduced since morphologically the chromosomes of the twelve forms studied are all similar in later stages, while at this period they are greatly lengthened because they have not yet arrived at their definitive size and shape. This fact would make futile any attempt to determine size and shape differences.

Late prophase

The opening of the spireme following the second contraction seems to involve two factors, an increased and persistent condensation of chromatin within the spireme, accompanied by a slight shortening of the spireme. These, together, give rise to a much more thickened and shortened open spireme, in which the heterotypic chromosomes become visible through the formation of constrictions between them. These constrictions may completely cut off pairs of chromosomes, or they may be incomplete, leaving the pairs attached to one another end to end. The number of pairs cut off and the number which remain attached end to end provide many different arrangements which have been found to be more or less characteristic for each form thus far studied. The closed circles formed by the chromosome pairs that are not completely cut off, and the numbers of detached pairs of chromosomes for the twelve different forms studied are as follows:

Pedigree numbers	*Forms studied*	*Arrangement of chromosomes*
22126, 22236, 22237, 22239	*Oe. Lamarckiana*,	1 pair and circle of 12.
22218, 23138	*Oe.* mut. *pervirens*,	1 pair and circle of 12, or 7 pairs; former more common.
22278	*Oe. rubricalyx sulfurea*,	7 pairs.
22282, 23151	*Oe. grandiflora*, B.,	7 pairs.
23173	*Oe. franciscana sulfurea*,	7 pairs.
23183	*Oe. suaveolens* (yellow),	1 pair and circle of 12.

23184	*Oe. suaveolens* (sulfur),	1 pair and circle of 12.
2338	*Oe. pratincola*,	1 pair and circle of 12.
2332 (7), 2332 (25)	*Oe.* seg. *deserens*,	4 pairs and circle of 6.
22285	*Oe.* (seg. *decipiens* ×*grandiflora*)F_1	4 pairs and circle of 6.
23159	*Oe.* (seg. *decipiens* ×*grandiflora*)F_2,	4 pairs and circle of 6, or 7 pairs; latter more common.
22284	*Oe.* (*grandiflora*×seg. *decipiens*)F_2,	4 pairs and circle of 6, or 7 pairs; latter more common.
23155, 23156	*Oe.* (*grandiflora*×seg. *decipiens*)F_2	4 pairs and circle of 6.

This late prophase is a relatively short one in all of these forms. From the time of their first appearance up to and including the heterotypic metaphase the closed circles and diakinetic pairs of chromosomes persist. The individual chromosomes continue to contract up to and during the formation of the bipolar spindle. Very seldom are all the members of a circle found lying in one plane but commonly in different planes as indicated by the shadings given in the drawings. The nucleolus has usually disappeared entirely by this time and if present is found to be small and faint. Because of the many variations during this stage I shall briefly describe each form separately.

Figures 13 and 14 show an early and late condition of diakinesis of *Oe. Lamarckiana*. In figure 13 the twelve chromosomes of the closed circle are still in an elongated condition, some more so than others. In figure 14 they have become contracted to approximately the condition in which they are ordinarily seen. Two of the chromosomes in this figure show a slight tendency to what appears to be a precocious split which later closes to appear again at interkinesis, in preparation for the homotypic division. The twelve chromosomes composing the closed circle are present, while one chromosome of the diakinetic pair is not visible. The nuclear wall has disappeared and spindle fibers have made their appearance while the nucleolus is fast disappearing. This closed-circle arrangement of twelve chromosomes in *Oe. Lamarckiana* is characteristic of the numerous cells of this species which were studied.

Oe. mut. *pervirens* is assumed to be the same genetically as *Lamarckiana* except that it has no red pigment and shows a higher percentage of germination than *Oe. Lamarckiana*. Most of the plants examined showed a single diakinetic pair and a closed circle of twelve. In figure 20 the chromosomes in the closed circle have already arrived at approximately

their definitive size. The nuclear membrane disappears soon after the appearance of the spindle fibers within the nucleus. Figure 21 shows an entirely different condition, of seven diakinetic pairs. This was not a common condition but one that does sometimes occur.

Oe. rubricalyx sulfurea. In this hybrid derivative we find constantly a seven-pair arrangement. The chromosome group shown in figure 28 was found lying entirely outside of the nuclear membrane, possibly having been carried out by the knife. Four of the pairs remain joined together. This union later evidently breaks (figure 29) and the pairs are scattered on the newly formed spindle. The nucleolus appears as a small, very faintly stained body which will soon dissolve away.

Oe. grandiflora B is a species which shows seven pairs of chromosomes (figure 32). This fact, first pointed out by DAVIS (1909), can be verified by the appearance and arrangement of the chromosomes seen at the heterotypic metaphase which will be described later (figure 33). *Oe. grandiflora* is considered a relatively stable species whose homologous chromosomes pair with great regularity (DAVIS 1919).

Oe. franciscana sulfurea is a hybrid derivative of *franciscana* × *biennis* and is similar to *franciscana* genetically except for its sulfur-colored flowers. This also presents a seven chromosome pair arrangement (figures 38, 39). In figure 39 the chromosomes, although arranged in diakinetic pairs, are still in a roughened, elongated condition. In this cell only six pairs could be found, which of course would lead us to suspect that the other two chromosomes were outside the section and were also paired. The nucleolus was present in both cells, though brown and vacuolated in one where it evidently would soon disappear. CLELAND (1924) found one pair and a circle of twelve for a similar hybrid, using material secured from the experimental gardens of Professor B. M. DAVIS.[2] *Oe. suaveolens* (yellow) is a species which resembles *biennis* in its breeding behavior. Here we find one pair and a closed circle of twelve chromosomes (figure 41). The single pair is shown linked into the closed circle. The chromosome circle can here be seen arranging itself upon the spindle. The nuclear membrane and nucleolus are both gone.

Oe. suaveolens (sulfur) is a "mutant" from the wild-type yellow-flowered form. It also has one pair and a closed circle of twelve chromosomes (figures 43, 44). The nucleolus in each cell is vacuolated and will soon disappear.

[2] Since this was written CLELAND (1928) has also reported seven free pairs in *Oe. franciscana sulfurea* from SHULL'S cultures.

Oe. pratincola is a wild species which resembles *Oe. biennis* genetically in giving unlike reciprocal hybrids. This species shows one pair and a closed circle of twelve chromosomes. The nucleolus is light brown in color, doubtless due to its loss of chromatin material (figure 46). Figures 47 and 48 show late prophase or early heterotypic metaphase stages in which some of the chromosomes of the closed circles are arranging themselves in a regular zigzag as has been reported by CLELAND, the result apparently being an equal numerical distribution produced by adjacent chromosomes migrating to opposite poles. In figure 47 only those chromosomes of the closed circle whose connections are distinct are shown. Figure 48 shows ten of the twelve chromosomes which compose the closed circle.

Oe. seg. *deserens* is a homozygous segregate from *rubrinervis*. The chromosome arrangement in the material collected under this name is four pairs and a closed circle of six (figures 50, 51).[3] The closed circles of six were very constant features in all cells studied. All of the four diakinetic pairs were never present in one cell together with the closed circle of six; usually only two or three pairs could be found. Figure 50 shows a circle of six chromosomes and three diakinetic pairs. The remaining two chromosomes evidently represent the fourth pair not shown in the section. Many other cells indicated the presence of circles or chains although the full complement of fourteen chromosomes could not always be determined. The nucleolus in each instance is light brown in color. The chromosomes here are also in an extended condition which will contract to their definitive size by the time heterotypic metaphase is reached. CLELAND (1925) found this segregate had a complete pairing of the fourteen chromosomes.

Oe. (seg. *decipiens*×*grandiflora*)F_1 is a hybrid which shows four pairs and a closed circle of six chromosomes (figures 55, 56). Concerning the chromosome arrangements at diakinesis in the two parents of these F_1 plants and those which remain to be described, we know that *grandiflora* constantly has shown seven diakinetic chromosome pairs. So far as known, seg. *decipiens* has never been studied cytologically and it apparently possesses no lethal factors and is quite regular in its genetical behavior. Four individual pairs, and never any more, was a very constant feature of cells of the F_1 plants, although the closed circle did not always

[3] Dr. SHULL advises me that *Oe.* seg. *deserens* which I have studied may have been wrongly identified, as the distinction between *deserens* and *rubrinervis* is not sharp, and such errors of classification have frequently occurred. CLELAND finds the same chromosome complement for *rubrinervis* that I have found for this supposed *deserens*.

appear in its complete form; while in other cells the closed circle of six chromosomes was constantly present, variations were observed in the number of the individual pairs. In figure 55 is seen the full complement and here the chromosomes, at least those in the closed circle, are still elongated. In figure 56 two adjacent chromosomes of the circle of six apparently are moving toward the same pole, but such behavior is very rarely seen.

Oe. (seg. *decipiens*×*grandiflora*)F_2 used in this study was not the following generation from the F_1 described in the last paragraph, but resulted from selfing an F_1 plant from a similar mating made a year earlier. Here we find two possibilities in the arrangement of the chromosomes. Only the most common chromosome arrangement (figures 61, 62),—seven pairs,—has been figured. The metaphase also shows the presence of such a seven-pair arrangement. Cells were repeatedly found, however, with four diakinetic pairs of chromosomes and an open circle of a variable number or a cluster of chromosomes which could not be individually distinguished. This alternative arrangement probably would consist of four pairs and a closed circle of six as was found to be the common condition in the F_1 plants of this cross.

Oe. (*grandiflora*×seg. *decipiens*)F_2. These are the F_2 plants of a cross reciprocal to the one presented in the last paragraph. Here also we find two possibilities, either seven individual pairs (figures 66, 67) which are united in various fashions or four individual pairs and a closed circle of six (figure 65). The six chromosomes of the closed circle are still quite well extended. The chromosome arrangement at metaphase (figure 69) also indicates a previous seven-pair arrangement. The occurrence of seven pairs is by far the more common. The chromosome arrangement is apparently identical with that in the corresponding generation of the reciprocal cross.

Another lot of *Oe.* (*grandiflora*×seg. *decipiens*)F_2 was found to present a somewhat different situation, the most common chromosome arrangement in this case being the one which was less common in the former case. The chromosome complement consists of four individual pairs and a closed circle of six (figures 70, 71). The nucleolus in each case is almost invisible or very light in color, indicating its rapid disappearance. These are all taken from smear preparations which are especially good.

The arrangements of the chromosomes given for the different species, crosses, segregates, and mutants are based upon numerous observations on hundreds of cells. All observations indicate that the chromosome arrangements are *relatively* constant for the particular forms studied, al-

though variations are sometimes seen. *Oenothera* mut. *pervirens* showed an alternative condition in the arrangement of its chromosomes. The alternative conditions found in *Oe.* (seg. *decipiens*×*grandiflora*)F_2 and *Oe.* (*grandiflora*×seg. *decipiens*)F_2 were perhaps to be expected, since the F_2 is normally a splitting generation. It should be recalled in this connection that the buds were selected from several plants of the forms studied and at least six successive collections were made in one season; also that wherever two different chromosome arrangements have been seen they have been found in different plants, and not in different figures of the same plant.

After the chromosomes had arrived at their definitive size, no characteristic morphological differences in size or shape could be determined, such as were reported by BOEDIJN (1924) for several Oenotheras, nor as reported by HANCE (1918) for *Oe. scintillans*.

Heterotypic metaphase and anaphase

During late prophase the chromosomes arrange themselves on the equator of the spindle in such a way that for the most part the metaphase and anaphase stages show a regular arrangement (figures 15, 16, 22, 42, 57, 69). If slight irregularities do occur, successive stages show equal numbers of chromosomes passing to each pole. In these twelve species, mutants and segregates, there was no evidence of unequal numbers of chromosomes at the heterotypic telophase or at interkinesis. There is no reason to believe that it never occurs, but the cytological evidence seems to indicate that it is at least a rare phenomenon.

The arrangement of the chromosomes at late prophase, metaphase, and early anaphase is of two general kinds, depending upon the presence of a closed circle or of diakinetic pairs. When a closed circle is present in the stages immediately following the opening of the second contraction we find the chain arranging itself in a way that usually would permit adjacent chromosomes to pass to opposite poles (figures 45, 47, 48), as first shown by CLELAND (1923). Figures 41, 44, 55, and 56 are slightly earlier stages which show the chains or circles arranging themselves in preparation for the stages described above. Due to the attachment of the spindle fibers at the middle of the chromosomes, as they are drawn to their respective poles, the chromosomes assume a V-shaped appearance. In a few cases no attachment is seen at the middle of the chromosomes, in which case the chromosomes lie fully extended and the characteristic V-shape is not seen. Perhaps this is due to attachments not yet having been made. This zigzag arrangement of the chromosomes which

compose circles of certain species is very significant because it results in an equal numerical distribution of seven chromosomes, the haploid number, to each pole. It seems probable, as CLELAND has argued, that this passage of adjacent chromosomes to opposite poles separates the members of homologous pairs, so that each daughter nucleus will receive a complete complement of haploid chromosomes. EMERSON (1925) alone of recent investigators on Oenothera did not find in his studies of *Oe. biennis*, any distribution of the chromosomes to the two poles in such a manner that homologous ones passed to opposite poles. The chromosome number at the heterotypic telophase is very constantly the normal haploid number—in fact no other condition was observed in these twelve forms. GATES (1907) first observed an unequal distribution to the daughter nuclei; CLELAND (1924) shows an instance in *Oe. franciscana sulfurea* of an unequal numerical distribution of 8 and 6 chromosomes respectively; and we know from the frequency of occurrence of 15-chromosome mutations (trisomic) in *Oe. Lamarckiana* that such irregularities are fairly frequent in that species. The regularity of the zigzag arrangement of the chromosome circles seems to justify the conclusion that these closed circles are composed of successive pairs of homologous chromosomes in which there is either a regular alternation of maternal and paternal chromosomes, as held by CLELAND, or more probably a chance arrangement of the maternal and paternal member of each pair, as recently proposed by SHULL (1928). SHULL is inclined to believe that the "univalent spireme of the Oenothera type is made up of a succession of pairs of homologous chromosomes in which the order of the individual chromosomes with respect to their maternal and paternal origin is indeterminate." The orientation of the chromosomes of each pair is thus a matter of chance and their distribution to their respective poles would likewise be purely a matter of chance, as happens when diakinetic pairs are formed. Closed circles in my material have so far revealed chromosomes only in even numbers and this accords with the view that the chromosomes maintain their association in pairs, but gives no evidence on the critical question as to the determinate or indeterminate position of maternal and paternal chromosomes in each pair.

The formation of diakinetic pairs (figures 33, 62, 69) of homologous chromosomes has been assumed by most writers to result from a certain degree of compatibility or affinity due to the similarity of constitution of the two chromosomes of each homologous pair, and this is perhaps true regardless of whether they previously had assumed a telosynaptic

or a parasynaptic arrangement. Since homologous chromosomes are assumed to play essentially the same rôle in the life of the organism, their genic constitution may be reasonably assumed to be either identical or relatively similar. There is no reason to think that the Oenotheras are an exception in this respect. In figures 33, 62, and 69 there is evidence from the arrangement of the chromosomes that they existed in individual diakinetic pairs during late prophase. Closed circles at this stage never show such pairing but rather the zigzag arrangement. It should be noted that in some cases (figures 15, 33, 69) one side of a diakinetic pair breaks before the other as they are drawn apart and pass toward their respective poles, resulting in two chromosomes attached end to end. The broken ends point toward opposite poles. This is a common feature of those species which are represented by diakinetic pairs and is especially well shown in *Oe. grandiflora*. The persistent attachment of such pairs is probably the original attachment in the spireme, while the broken attachment is probably the new attachment made in diakinesis.

In both cases, we find the chromosomes passing rapidly toward their respective poles. A splendid example of the regular behavior of the chromosomes at mid-anaphase is to be seen in *Oe. franciscana sulfurea* (figure 40).

Heterotypic telophase and interkinesis

The chromosomes seen at telophase show signs of splitting in preparation for the homotypic mitosis. This is the usual time for such splitting to occur in the Oenotheras studied. The splitting begins at both ends (figure 23). The nucleus that is first formed is small, but during interkinesis it grows to be at least 9.18μ in diameter. During this growth period the chromosomes continue splitting until they lie back to back touching in their middle with their ends curved away from each other, or they may lie connected at one end, the other ends lying free (figures 17, 18, 23). In some cases they resemble a maltese cross (figure 23). Six of these split chromosomes are generally seen to arrange themselves around the periphery of the nuclear membrane while the seventh occupies a central position. A nucleolus also soon appears, apparently *de novo*, and rapidly increases in size (figures 17, 18). The interkinetic stage is a relatively long one making it easy to find numerous stages.

The chromosomes soon become irregular and roughened along their edges; the nuclear membrane dissolves and spindle fibers begin to be laid down preparatory for the homotypic mitosis.

Homotypic mitosis

As homotypic prophase approaches, the chromosomes assume a small dumb-bell-shaped appearance (figure 24). The spindle is tripolar at first as in the heterotypic prophase but soon becomes bipolar. Progress from prophase to metaphase is very rapid. The chromosomes quickly arrange themselves on the spindle and at metaphase we find them in a very regular order (figure 25). This regularity is quite general for all these twelve forms, and irregularity is an exception. This division is evidently an equational one.

The chromosomes at each heterotypic telophase pass through the same stages until finally the cell contains two homotypic spindles. These may lie in the same plane (figure 26) but more generally at right angles to each other (figure 19). These latter are drawn as though they were in one plane while in fact a considerable change of focus was necessary in order to see the four nuclei. Their position also would indicate that the two spindles were perpendicular to each other. Figure 26 is an earlier stage than figure 19; the chromosomes are small but still distinguishable. In figure 19 a nuclear membrane and nucleolus have appeared and the chromosomes appear to be fast losing their individuality in a fine network of fibers.

The cell body prepares to divide at this time to give rise to four granddaughter cells or microspores. Both figures 26 and 19 show the beginning stages of furrowing which proceeds until the four cells with their respective nuclei are cut off. No cell plate was seen as a preliminary step in the division of the pollen mother cell. The walls of the four resulting granddaughter cells continue growing in thickness until they have assumed their characteristic spore appearance.

DISCUSSION

A study of these twelve Oenothera species, segregates, mutants and hybrid progenies is of particular interest in relation to two important periods of meiosis in the pollen mother cells, namely, synizesis, especially the second contraction stage, and late prophase, when chromosome pairs and closed circles are in the process of formation. In reference to synizesis there are two points to be considered, (1) the presence of a continuous spireme consisting of a series of univalent chromosomes in telosynapsis, and (2) the fact that the second contraction period provides for the mechanical process of crossing over.

In reference to the late prophase period there are the following questions

to be discussed, (1) whether closed circles are constant and characteristic for the species concerned, (2) whether the diakinetic pairs are due to the homozygous character of the chromosomes, and non-paired chromosomes or closed circles to the heterozygous constitution of the chromosomes, as assumed by CLELAND, (3) whether the homologous chromosomes of closed circles are more probably indeterminate in their arrangement, with consequent independent assortment, as assumed by SHULL, or determinate in arrangement as assumed by CLELAND, and (4) whether closed circles bear any relation to linkage groups.

Synizesis

Evidence that synizesis is a normal process in spireme formation and not an artifact may be seen in the fact that early and late stages of synizesis, late heterotypic prophase and metaphase stages have all been found in one bud, while the first contraction and open spireme stages have been constantly associated together in the same loculus. All observations indicate that synizesis is the process whereby the definitive spireme is formed. Numerous observations were made on all stages of spireme formation from and including the archesporial cells to the time of diakinesis, and both monocular and binocular lenses were employed with both artificial and natural light. Although particular attention was given to securing reliable data upon this point, I could find no evidence of a bivalent spireme whose sides represent the halves of future homologous chromosomes, nor of a split univalent spireme, but in all the Oenotheras here studied, only a continuous, evidently univalent, spireme was seen.

The two contraction periods appear to involve two processes, condensation and contraction. The linin fibers which eventually make up the spireme thread are probably contractile in nature; and the chromatin material throughout early stages is partly scattered in the nucleus and partly stored in the nucleolus. Consequently, as condensation and contraction proceed, they result in chromatin being laid down on or within the linin fibers. Both processes may be assumed to go on simultaneously but at varying rates. The contractile power of the linin fibers produces a tightly contracted mass, the first contraction; then condensation continues to take place in the laying down of more chromatin and the contraction mass is pushed open, even though some contraction may still continue. This results in the open spireme stage when the spireme is for the first time easily identified as a continuous univalent structure (figure 59). When fully unfolded, a short period of balance is apparently attained between the two processes,—condensation and contraction. Since

contraction is still in progress and condensation apparently has spent its force for the time being, in fully unfolding the spireme, the natural consequence is a second contraction stage. Condensation, being still incomplete, sets in again, resulting finally in a complete spireme. Such a continuous univalent spireme at this stage has been reported by GATES (1908, 1910), GEERTS (1908), DAVIS (1911) and CLELAND (1922, 1924, 1926a, 1926b) on the Oenotheras. Oenothera is not unique in this respect, however, as MOTTIER (1909) in *Lilium Martagon* and DIGBY (1919) in Osmunda have reached similar conclusions, except that a longitudinal split was observed which was interpreted as a split univalent spireme. MOTTIER and DIGBY also hold that the bivalent chromosomes arose during the second contraction by looping as seen in the Oenotheras.

In connection with the significance of synizesis must be considered the prevalent method of telosynapsis or end to end arrangement of the chromosomes. Of all the workers on Oenothera, only BOEDIJN (1924) doubts the telosynaptic arrangement of the chromosomes. This phenomenon of telosynapsis or end to end arrangement of the chromosomes depends upon the earlier presence of a continuous univalent spireme. The persistent loops seen at second contraction are preliminary evidence of the formation of chromosome pairs from such a univalent spireme. Further evidence is seen at the time the second contraction mass begins to open. The univalent spireme, upon being released from the second contraction, is undergoing segmentation or constriction in the visible formation of the heterotypic chromosomes. In some cases adjacent chromosomes will pair (figure 36), giving rise to seven chromosome pairs as seen in three of the twelve forms studied, *rubricalyx sulfurea*, *grandiflora* and *franciscana sulfurea*, and as seen in two others, *Oe.* (*grandiflora* × seg. *decipiens*)F_2 and *Oe.* (seg. *decipiens* × *grandiflora*)F_2, as the more common condition of two alternatives. Also in *Oe.* mut. *pervirens* this may sometimes be seen. In other cases some of the chromosomes persist in hanging together to form closed circles, (see page 612). These closed circles were found quite constantly in seven of the forms studied, and also, but less constantly, in the three forms mentioned above in which some plants were found with all the chromosomes paired. These closed circles consist of six chromosomes in some forms and twelve chromosomes in others. There seems to be no doubt about their arrangement end to end in a telosynaptic fashion.

A complete historical discussion of telosynapsis will not be given here. Reference need only be made to the general discussion by FARMER (1912) and to the recent work of GATES, DAVIS and CLELAND on Oenothera.

A significant fact about the second contraction stage is the peculiar

twisting of the loops and apparent fusion at points on the sides of these loops. The loops vary in number and do not necessarily correspond to the number of chromosomes. It therefore might be argued that these loops, with their twisting and apparent fusions, are nothing but the natural results of a condensation and thickening of the spireme thread which, upon being strongly contracted, throws a few loops, some of which may twist and approximate each other closely; but my observations lead me to believe that these loops are usually made up of pairs of homologous chromosomes, and for the following reasons:

During the opening of second contraction, it is seen that segmentation of the spireme is taking place (figures 12, 49) and the ends of some of the loops represent places of segmentation. This is not always the case, however, and the segmentation is sometimes seen on the sides of the loops as pointed out by HÅKANSSON (1926). In some cases the loops become actually detached from the rest of the spireme as separate chromosome pairs and in these cases it is practically certain that the two sides of the loops are made up of a pair of homologous chromosomes. It seems probable that the sides of the loops generally have the same significance when they do not become detached but remain as parts of a multisomal circle (figures 31, 35, 36, 49).

This is the first stage, moreover, in which the individual chromosomes can be recognized. The chromosomes are long, extended, roughened structures which have not yet arrived at their definitive size and shape. If the twisting and apparent fusions of the sides of a loop should result in the halves of the loop breaking so that parts of each half unite to form each of two new chromosomes, a mechanism would thus be provided for the crossing over that the genetical evidence demands. There is no direct cytological evidence that such interchange of parts between homologous chromosomes actually takes place, although figures 27 and 31 and one pair of chromosomes in figure 37 are suggestive in this respect. Neither would this phenomenon be expected to result in crossing over in every case where twisting and apparent fusions occur. My observations on this point were first made in 1923.

GATES and REES (1921), in the study of Lactuca, observed a twisting and an untwisting of the sides of such projecting loops and in some cases there was some evidence that breaks occurred where twisting had taken place. BOEDIJN (1924) in studying the heterotypic cell divisions in Oenothera finds the loops of the second contraction particularly interesting inasmuch as he holds that crossing over must occur at this stage. Miss J. LATTER (1925) in studying the pollen development in the sweet-

pea makes similar observations of long slender loops which twist about each other near the base. She concludes that this would provide a possible basis for crossing over in cases of telosynapsis. CLELAND (1926a) in his study of *Oenothera biennis* and *Oenothera biennis sulfurea* points out the possibility of this stage offering an opportunity of chromatic exchange in crossing over, but on the other hand argues in favor of what he calls "interchromosomal crossing over."

Nothing more conclusive cytologically can be said concerning crossing over in Oenothera at the second contraction stage, except that it offers a mechanical process by which crossing over, as already demonstrated through genetical evidence by SHULL (1923a, 1923b, 1927), may *possibly* take place. This provides a method for the exchange of inheritance factors or genes between homologous chromosomes that exhibit closed circles as well as those which constantly show diakinetic pairs, and negatives GATES's (1922) statement that no mechanism exists in Oenothera by which rossing over may be brought about.

Significance of chromosome circles

In reference to the first point concerning closed circles, it will be recalled that I have found two alternative conditions of chromosome arrangement in three different forms. Two of these are the crosses *Oe.* (seg. *decipiens*×*grandiflora*)F_2 and *Oe.* (*grandiflora*×seg. *decipiens*)F_2. The more common condition in both of these crosses is the seven-pair arrangement as in *grandiflora* although there was also found an arrangement of four pairs and a circle of six. Lethals evidently are not involved in this case, since seg. *decipiens* is supposed to be free of lethals and, so far as known, *grandiflora* is also. These observations were made on different plants. This case then possibly represents a segregation with respect to chromosome arrangement, a condition which might well be expected. Two different conditions were also found in *Oe.* mut. *pervirens*. Here seven individual pairs were found in some individuals but the more common configuration was one pair and a circle of twelve. CLELAND (1924) found for *Oe. franciscana sulfurea* from DAVIS's material a circle of twelve chromosomes and one diakinetic pair, while I have found constantly in SHULL's cultures of this form seven individual pairs. CLELAND has also found seven individual pairs in SHULL's cultures.[4] Again, for *Oe.* seg. *deserens*, a relatively stable segregate from *Oe. rubrinervis*, CLELAND found in plants from seed secured from Professor DE

[4] Reported in a letter to Doctor SHULL.

VRIES, seven complete pairs, while I have found in material from SHULL's cultures only four pairs and a circle of six. (Attention here should be drawn to a previous note, page 615, regarding the possibility of the *Oe.* seg. *deserens* I have used having been wrongly identified. Too much emphasis, therefore, should not be placed upon this result.) This makes a total of five out of twelve species and hybrids investigated, each of which has definitely shown alternative chromosome arrangements. This, then, suggests a probably affirmative answer to the question that SHULL (1924) asks, "Do different strains of *Lamarckiana* differ in the grouping of the chromosomes?" It is not a direct answer for *Lamarckiana* but since *Oe.* mut. *pervirens* is so similar to *Lamarckiana*, and two different configurations have been found for *pervirens*, it may be that *Lamarckiana* will show similar variations in chromosome grouping. However, a considerable number of plants of *Oe. Lamarckiana* from SHULL's cultures have been examined both by CLELLAND and by myself, but no plant of this species has yet been found which did not show the circle of twelve and one independent pair.

A recent paper by Miss SHEFFIELD (1927) shows figures which are considered to be unusual chromosome configurations, in addition to the more common and presumably constant arrangement, for three out of five Oenothera species and mutants studied by her. Emphasis is placed upon the more constant arrangement in each case while the unusual or alternative condition is dismissed as being "abnormal."

The three forms showing an unusual or alternative chromosome arrangement are: *Oe. novae-scotiae*, *Oe. rubricalyx* and *Oe. Agari*. *Oe. novae-scotiae* is a segregate from *Oe. muricata* which has bred true for many years. Collections were made from several plants. The more constant arrangement was a closed circle of fourteen, while the unusual or alternative arrangement was closed circles of six and eight, respectively. Another, *Oe. rubricalyx*, is a mutant which appeared in a culture of *Oe. rubrinervis* in 1907. Collections were from different plants. The more constant arrangement of the chromosomes was four pairs and a closed circle of six. *Oe. Agari* is a species which has been extremely uniform in appearance and behavior except for the occurrence of one narrow-leaved mutant. The more constant arrangement was a closed circle of fourteen, while the unusual or alternative condition consisted of several closed circles. This study of Miss SHEFFIELD'S, then, would add three Oenothera species and mutants with alternative chromosome configurations to the five already enumerated.

The evidence therefore would appear to indicate that closed circles of

any particular number of chromosomes are not constant as held by CLELAND (1923, 1924), but only *relatively* constant features for the individual species concerned. It should be noted also that in no case where chromosome configurations differ within the same phenotype has there been recorded any corresponding particular genetical variation which might be associated with the observed cytological differences.

In reference to the next point, it is generally recognized that only homologous chromosomes, which are supposed to play, in general, the same rôle in the life of the organism, pair at diakinesis. An "affinity" or "compatibility" between homologous chromosomes, enabling them to form synaptic pairs or even larger groups, as triploids, tetraploids, etc. at diakinesis, has been assumed by many investigators. A full discussion of this point is given by SHARP (1926) in which he concludes, page 282, "very little is known about the factors which bring about the synaptic union, but there is much evidence that the mutually attractive homologues show similar structural characteristics, and the genetic data indicate a corresponding of hereditary function also." DAVIS (1909) in his study of *Oe. grandiflora* noted that incompatibility between homologous chromosomes was lacking. The homologous chromosomes paired regularly and subsequently behaved in a very typical manner. This species is extremely stable, genetically, and shows very little pollen-and seed-sterility. CLELAND (1922) in his study of *Oe. franciscana* assumes that the failure of homologous chromosomes to pair at diakinesis and their tendency to come to the equatorial plate in an irregular fashion are characteristic of not all of the Oenotheras but only of those species which are recognized as genetically unstable. He also assumes that in stable species the homologous chromosomes appear to have a strong affinity for one another at diakinesis, and that any lack of affinity or incompatibility is due to the hybrid nature of the species. CLELAND (1926) again presents this theory in his study of *Oe. biennis sulfurea* as an explanation of the lack of chromosome pairing at diakinesis. In a recent work of HÅKANSSON (1925) on *Godetia amoena* and *Godetia Whitneyi* parasynapsis seems to be the rule and the chromosomes ordinarily pair at diakinesis. But in the hybrid *G. amoena*×*G. Whitneyi*, HÅKANSSON finds a general lack of pairing at diakinesis and in some cases the chromosomes are attached end to end as in Oenothera. He feels that the lack of pairing and the union of chromosomes end to end in Oenothera possibly came about originally through hybridization.

Since no particular genetical variations thus far have been reported in those forms which have shown two different chromosomal configurations,

as, for example, *Oe. pervirens* which sometimes gave seven complete pairs and more often a circle of twelve and one pair, it would seem doubtful whether we could assume the chromosomes in the latter case to be heterozygous simply because twelve of the chromosomes sometimes remain attached end to end in a closed circle. Again, stable genetical behavior apparently has no relation to the chromosomal configurations, for *Oe. franciscana* is a very stable species and has been shown to have five pairs and a circle of four chromosomes; *Oe. muricata* on the other hand, is also very stable genetically and has a circle of fourteen chromosomes; while *Oe. grandiflora* B is a very stable species and shows seven synaptic chromosome pairs.

Future studies may reveal genetical differences between two plants of some given species which have different chromosomal configurations, but until then I would not attempt to explain closed circles on the basis of heterozygosity. I would assume rather that all the diploid chromosomes of Oenothera may be homozygous or heterozygous, or some may be homozygous and some may be heterozygous, depending upon the known or discoverable behavior of the plants concerned, when subjected to appropriate genetical analysis.

Without attempting a full explanation of closed circles composed of chromosomes attached end to end, I would suggest that such nuclear structures may possibly be the result of the presence of certain genetic factors or combinations of factors, just as other physical characters of the phenotype are known to be produced. This idea is suggested by the chromosomal configurations exhibited by *Oe.* (seg. *decipiens*×*grandiflora*) F_2 and *Oe.* (*grandiflora*×seg. *decipiens*) F_2, which, as already pointed out, possibly represent a segregation with respect to chromosome arrangement.

Unfortunately, in the chromosomes of Oenothera, at least in those under investigation, shape and size differences of any kind which could offer additional evidence concerning their maternal or paternal origin and arrangement in the spireme, were not found. Several investigators have reported chromosome size differences in some of the Oenotheras. HANCE (1918) in *Oe. scintillans* found that a member of a pair differs from one of the next shorter pair by about 9 percent of its length. In this connection it should be pointed out that individual chromosomes were not identified by HANCE, but only inferred from measurements on enormously enlarged camera lucida sketches. Recently BOEDIJN (1924) working on *Oe. Lamarckiana* claims to have seen one very large chromosome pair, two nearly as large and four which are smaller.

The chromosomes of any homologous pair, nevertheless, must lie

adjacent to each other in the spireme; otherwise the pairing and regular separation of homologous chromosomes would be impossible unless we might assume that the chromosomes, when formed at the time of second contraction, break apart individually and are reassembled in pairs or circles at a later stage. But observations give no support for such an assumption.

Telosynaptic union of the chromosomes in a closed circle may consist of an alternate maternal and paternal arrangement throughout the circle as assumed by CLELAND, or in a manner suggested by SHULL (1928), who argues in favor of an indeterminate arrangement of the individual chromosomes of any homologous pair. CLELAND'S determinate arrangement, SHULL points out, presents features not in harmony with the known genetical evidence. SHULL assumes that the spireme or closed circle consists of a succession of pairs of homologous chromosomes in which the order of the individual chromosomes in regard to their maternal or paternal origin is reversible; that is, that the "orientation of the chromosomes of each pair is a matter of chance, so that in following a series of spiremes from left to right any chromosome of maternal origin will lie to the left of its paternal homologue in 50 percent of the cases, on the average, and to the right in the remaining 50 percent." Thus, for example, if we let capital letters stand for maternal chromosomes and small letters for paternal chromosomes there might be spiremes as follows: Aa, Bb, cC, dD, Ee, Ff, Gg, and aA, Bb, Cc, dD, eE, fF, Gg, and so on. On the other hand, the arrangement according to CLELAND'S hypothesis is Aa, Bb, Cc, Dd, Ee, Ff, Gg. It is necessary to assume the latter or determinate arrangement in order to secure true-breeding species, *if*, as CLELAND maintains, closed circles are constant and characteristic for each particular species, and *if* they are composed of chromosomes of heterozygous constitution. As already pointed out, I have found two different arrangements of the chromosomes in each of several forms of Oenothera, which forces me to conclude that closed circles are only relatively constant. I have also taken the attitude that all of the chromosomes of Oenothera may be homozygous or heterozygous, or some may be homozygous and others heterozygous depending for this viewpoint upon their genetical behavior when lethals are absent, and that closed circles possibly have no relation to the heterozygosity of the plant. CLELAND'S determinate arrangement precludes any independent assortment of the chromosomes unless they form one or more free synaptic pairs. On the other hand, the chance arrangement of the chromosomes of each pair in the spireme, as suggested by SHULL, permits, through their zigzag movement in early anaphase,

independent assortment of the chromosomes in Mendelian ratios such as is characteristic of most other plants and animals thus far studied. With either arrangement the unique genetical behavior of the Oenotheras requires the assumption of lethal factors or lethal combinations.

Investigations on Drosophila and other organisms have shown clearly that the number of linkage groups corresponds to the number of diploid pairs of chromosomes. In other words, a linkage group of factors is located in a single chromosome pair (or disome). So far, in *Oenothera Lamarckiana*, three linkage groups have been discovered (SHULL 1923a, 1923b, 1925, 1926, 1927), which at once postulates the necessity of at least two free synaptic pairs of chromosomes for *Lamarckiana*, *if* the homologous pairs in closed circles are arranged in the determinate fashion of CLELAND. But since *Lamarckiana* has so far shown only one pair and a circle of twelve chromosomes, and according to CLELAND'S hypothesis the circle of twelve chromosomes provides the basis of a single linkage group, the number of linkage groups of this species would be limited to two, and the question naturally arises, where, on this basis, would the new linkage group III, with *vetaurea* as the marker, be placed. *Brevistylis* is the marker for group II.

If we should assume an indeterminate arrangement of the homologous chromosomes within the pairs which compose the closed circles, a free assortment of these chromosomes at maturation would result, in spite of their union end to end, and these pairs would therefore provide the basis of a linkage group as is known to be the case for all other organisms sufficiently studied. There should be, on this assumption, a possibility of seven linkage groups, only three of which have yet been identified.

Although, to date, no *Lamarckiana* has been found which did not have twelve chromosomes joined in a circle, my results suggest that some other configuration may sometimes occur. No plant of this species that has served as a parent of a splitting progeny has yet had its chromosome configuration determined. The discovery of a *Lamarckiana* with a closed circle of twelve chromosomes and one pair, that simultaneously segregated factors for the second and third linkage groups, would definitely refute CLELAND'S hypothesis that closed circles provide the bases for single linkage groups; but until such an individual is observed, or other similar evidence becomes available, the question remains an open one.

SUMMARY AND CONCLUSIONS

1. Cytological studies were made on the pollen mother cells of twelve species, mutants and hybrids of Oenothera from the pedigree cultures of Professor G. H. SHULL.

2. At no time during the resting and growth period was there any evidence of parallelism among the spireme threads which might be attributed to a longitudinal splitting or parasynapsis of two univalent spiremes.

3. Two processes, contraction of the linin threads and condensation of the chromatin material, are thought to be involved in synizesis.

4. Rapid nuclear growth takes place at this stage and the movement of the reticular threads and their position at one side of the nucleus may be due to pressure exerted on them by the incoming cell sap.

5. No indications of fusion of separate spireme threads were seen. The spireme seems to be fused with the nucleolus, and to serve as an avenue for the exit of chromatin material from it.

6. As the spireme develops the nucleolus becomes lighter-staining and more or less vacuolated.

7. The contraction stages are believed to be normal, and not to be artifacts produced by the process of fixation.

8. In second contraction, loops varying in size and number project from the contracted mass. These probably represent usually, but not always, pairs of homologous chromosomes.

9. These loops have been seen twisted about each other in a manner which suggests that they provide a mechanism for an exchange of segments of homologous chromosomes, which could result in crossing over of genetic factors.

10. In late prophase the chromosomes attain their definitive size and shape, and either remain attached end to end in closed circles, or some or all form separate pairs.

11. *Oenothera Lamarckiana* has twelve chromosomes in a circle and one independent pair.

12. *Oe.* mut. *pervirens* showed two alternate configurations, the more common being like *Oe. Lamarckiana*, with a circle of twelve and an independent pair. Less frequently all the chromosomes form free pairs.

13. In *Oenothera rubricalyx sulfurea* all the chromosomes were found in diakinetic pairs.

14. *Oe. grandiflora* B was found to have seven pairs as reported by DAVIS.

15. *Oenothera franciscana sulfurea* has likewise seven pairs, in contrast to the corresponding form from DAVIS'S cultures in which CLELAND reported a circle of twelve and one pair.

16. *Oenothera suaveolens*, both yellow-flowered and sulfur-flowered strains, have twelve chromosomes in a closed circle and one free pair.

17. *Oenothera pratincola* likewise has one free pair, and twelve chromosomes united in a circle.

18. In *Oe.* seg. *deserens* a circle of six and four pairs was found, but as this was the reported configuration for *Oe.* mut. *rubrinervis* with which *deserens* is occasionally confused, it seems reasonable to suppose that the form examined under the name *deserens* may have been a *rubrinervis*.

19. The F_1 hybrids between *Oe. grandiflora* and *Oe.* seg. *decipiens* showed constantly a circle of six and four pairs.

20. The F_2 from the last-mentioned cross showed two configurations in different plants, in some a circle of six and four pairs, in others seven pairs, suggesting a definite segregation with respect to the chromosome configuration. This was true in both reciprocal combinations.

21. No differences were observable in size or shape of chromosomes after they had reached their definitive condition.

22. Chromosomes included in the closed circles are even in number and separate in anaphase alternately to opposite poles. No cases of unequal numbers going to the two poles were seen, though the frequency of occurrence of trisomic mutants indicates that such unequal distribution is of not infrequent occurrence.

23. In interkinesis the chromosomes usually split from each end and the ends curve away from each other presenting the form of a maltese cross, but sometimes the split takes place only at one end.

24. As homotypic prophase approaches the chromosomes assume the form of small dumb-bells. The homotypic spindle is at first tripolar but soon becomes bipolar. The two homotypic spindles usually form at right angles to each other.

25. Since forms phenotypically alike have been shown in a number of cases to present alternative chromosome configurations, and no close correspondence between genetical phenomena and chromosome configuration has been demonstrated, it may still be doubted whether the multisomal circles have fundamental genetical significance.

26. The assumption that chromosomes are joined end to end because of their heterozygosity is not yet indicated by any genetical experience, and is of doubtful validity. The physical condition of the linin framework of the spireme which prevents the chromosomes from separating in heterotypic prophase may be controlled by specific genetic factors, just as other phenotypic characteristics are determined.

27. No cytological observations have given assistance in deciding the determinate or indeterminate position of paternal and maternal chromosomes in the homologous pairs which compose the multisomal circles.

The occurrence of three linkage groups in *Oe. Lamarckiana*, in which only one independent pair of chromosomes has yet been observed, is favorable to an indeterminate arrangement of the members of at least one of the pairs of homologs included in the circle.

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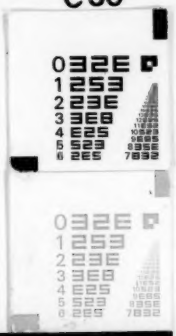
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